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Synthesis and Biochemical Activities of Antiproliferative Amino Acid and Phosphate Derivatives of Microtubule-Disrupting β-Lactam Combretastatins

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Abstract

The synthesis and biochemical activities of novel water-soluble β-lactam analogues of combretastatin A-4 are described. The first series of compounds investigated, β-lactam phosphate esters **7a**, **8a** and **9a**, exhibited potent antiproliferative activity and caused microtubule disruption in human breast carcinoma-derived MCF-7 cells. They did not inhibit tubulin polymerisation *in vitro*, indicating that biotransformation was necessary for their antiproliferative and tubulin binding effects in MCF-7 cells. The second series of compounds, β-lactam amino acid amides (including **10k** and **11l**) displayed potent antiproliferative activity in MCF-7 cells, disrupted microtubules in MCF-7 cells and also inhibited the polymerisation of tubulin *in vitro*. This indicates that the β-lactam amides did not require metabolic activation to have antiproliferative effects, in contrast to the phosphate series. Both series of compounds caused mitotic catastrophe and apoptosis in MCF-7 cells. Molecular modelling studies indicated potential binding conformations for the β-lactam amino acid amides **10k** and **11l** in the colchicine-binding site of tubulin. Due to their aqueous solubility and potent biochemical effects, these compounds are promising candidates for further development as microtubule-disrupting agents.

Keywords

Antiproliferative; beta-lactam; combretastatin A-4; prodrug; solubility; tubulin.

Abbreviations

CA-1	Combretastatin A-1
CA-4	Combretastatin A-4
DIPEA	Diisopropylethylamine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC N-Acetylcolchicine

PBS Phosphate buffered saline

1. Introduction

Solubility limitations to oral drug delivery are frequently encountered in drug development [1, 2]. There are many formulation and drug design strategies that can be used to overcome solubility issues, such as use of surfactants and co-solvents. Design of soluble prodrugs is a routine technique used to overcome solubility problems [3]. We have recently reported a series of β-lactams with extremely potent antiproliferative effects [4, 5]. These compounds are amongst the most potent analogues of combretastatin A-4 reported to date, but their further development is limited by lack of aqueous solubility. Hence, we designed phosphate- and amino acid-containing derivatives of the β-lactams to improve their pharmacokinetic profile.

1.1 Phosphate ester prodrugs

Phosphate ester prodrugs are useful for phenolic and amino-containing poorly water-soluble drugs to enhance their aqueous solubility. Phosphate prodrugs display excellent chemical stability and rapid bioconversion *in vivo* to the parent drug by phosphatases present in the intestinal brush border or in the liver [1]. Examples of commercially available phosphate prodrugs include the anti-epileptic fosphenytoin [3], the antiviral fosamprenavir and the steroid prednisolone phosphate [1]. Phosphate prodrugs of tubulin-binding agents are known, for example those of *N*-acetylcolchicine (**1**), combretastatin A-4 (**2**), combretastatin A-1 (**3**) and phenstatin (**4**, Figure 1). Phosphate prodrug **1a** (Figure 1) demonstrated increased water-solubility and delayed metabolism compared to **1**.

Analogue **1a** showed comparative effects to **1** *in vivo* but did not inhibit *in vitro* polymerisation of tubulin, indicating that phosphate compound **1a** itself is inactive and requires enzymatic activation *in vivo* [6]. Prodrug **1a** was in clinical trials in the United States for the treatment of metastatic renal cell carcinoma, but these were halted due to cardiotoxicity problems [7].

Combretastatin prodrugs have been extensively investigated, including phosphate prodrugs of combretastatin A-4 (CA-4, **2**, Figure 1) [8], combretastatin A-1 (CA-1, **3**, Figure 1) [9], combretastatin B-1 [9], stilstatins 1 and 2 [10] and iodocombretastatins [11]. Due to the sparing aqueous solubility of **2**, initial formulation attempts were unsuccessful [8]. The phosphate ester prodrug CA-4P (**2a**, Figure 1) had excellent water solubility, good stability and good cell growth inhibitory activity, with a mean panel GI_{50} of 6.89 nM (compared to 6.61 nM for **2**) in the NCI screen [8]. Prodrug **2a** is rapidly dephosphorylated to **2** *in vivo* with a half-life of a few minutes [12]. It is under evaluation in phase 3 trials for treatment of anaplastic thyroid cancer and in phase 2 trials for non-small cell lung cancer and platinum-resistant ovarian cancer [13]. Phenstatin (**4**, Figure 1) is a benzophenone-type compound structurally similar to **2** with potent antiproliferative and tubulin-binding activity but poor aqueous solubility. A phosphate prodrug (**4a**, Figure 1) was prepared to improve water-solubility and had equivalent antiproliferative activity compared to the parent compound **4** [14].

1.2 Amino Acid Prodrugs

Like phosphate prodrugs, amino acid prodrugs also confer the advantage of increased aqueous solubility to poorly soluble drug molecules. Additionally, drugs with an amino

acid residue may be substrates for peptide transporters, allowing them to be taken up by endogenous transporters in the intestinal epithelium [1]. Of a series of amino acid prodrugs of CA-4 amino analogue **5** (Figure 2), serine derivative **5a** retained potent antiproliferative activity (IC_{50} value of 27.2 nM in colon 26 cells compared to 2.8 nM for **5**) while improving the solubility in human plasma compared to **5** from 1.4 mg/mL to 3.3 mg/mL [15]. Prodrug **5a** is in clinical trials for advanced-stage soft tissue sarcoma, solid tumours and advanced solid tumours [16]. Amino acid prodrugs of combretastatin A-2 amino derivative **6** (Figure 2) have also been reported, with glycine and tyrosine amides possessing the most potent antiproliferative effects (mean IC_{50} value of 20 nM for glycine derivative **6a** and 17 nM for tyrosine derivative, compared to 13 nM for **6**, across a panel of six human cell lines). The amide prodrugs of **6** displayed little inhibition of tubulin polymerisation *in vitro*, indicating a requirement for bioactivation [17].

β-Lactams for further development were selected from our previous studies on the basis of their potency in antiproliferative assays and the presence of suitable functional groups (phenolic and amino) for modification (**7**-**11**, Figure 3) [4, 5, 18]. The aim of this work was to synthesise water-soluble β-lactams with potent antiproliferative effects for pharmaceutical use and to characterise their biochemical effects.

2. Chemistry

Phosphate esters of three potent phenolic β-lactams (**7**, **8** and **9**) [4, 5] were prepared in two steps (Scheme 1). Benzyl-protected compound **9c** was used to achieve selective esterification of the phenol at the C-4 position of **9**. Reaction with dibenzylphosphite was

achieved using diisopropylethylamine (DIPEA) and dimethylaminopyridine (DMAP) to give **7b**, **8b** and **9b** in yields up to 74% (Scheme 1, step *a*). Introduction of the dibenzylphosphite group to the phenol of the β-lactam was confirmed by the appearance of signals integrating for 4 protons at approximately δ 5.15 ppm in the ^{1}H NMR spectra of **7b**, **8b** and **9b**, attributable to the benzylic CH_2 protons. The final step was hydrogenolysis of the benzyl protecting groups to prepare **7a**, **8a** and **9a** in high yields (Scheme 1, step *b*). The deprotection of **7b** was initially achieved in 72% yield by treatment with bromotrimethylsilane. Debenzylation (H_2/PdC) of the β-lactam intermediates in high yield was successful without decomposition of the β-lactam ring.

Initial investigation into potential amino acid prodrugs of **7** were directed at preparation of amino acid esters. The Cbz-protected alanine ester **7e** and phenylalanine ester **7f** along with N-acetyl glycine ester **7g**, were obtained by DCC/DMAP coupling of the phenolic moiety of **7** with the appropriate amino acids (Scheme 2). Removal of the Cbz protecting group from **7e** afforded the alanine ester **7h**. However, isolation of the corresponding phenylalanine and glycine esters proved difficult due to very rapid hydrolysis to the phenol **7**. The preparation of a number of additional Cbz protected amino acid esters of **7** was investigated, but in each case the deprotected ester was rapidly hydrolysed. These esters are not suitable for further prodrug development. The acetate and benzoate esters **7i** and **7j** were prepared by Staudinger reaction of corresponding imines **12a** and **12b** with phenylacetyl chloride. A basic ether derivative **7l** was also synthesised (Scheme 2). The ether linkage would not be susceptible to hydrolysis *in vivo* while the basic side chain may improve the water solubility of the derivative. Reaction of the imine **12c** with 1,2-

dibromoethane afforded the bromide **12d**, which was then treated with phenylacetyl chloride to produce the β-lactam product **7k**. Reaction of the bromide **7k** with methylamine afforded the required product **7l** in low yield (30%) as the basic reaction conditions resulted in extensive cleavage of the β-lactam ring.

In order to prepare further amino acid β-lactams amides, amino-containing azetidinones **10** and **11** [4] were coupled to a series of protected L-amino-amino acids. DCC, with DMAP and HOBt.H_2O, was used to couple either **10** or **11** to the carboxylate moiety of amino acids to afford the products **10a-10f** and **11a-11h** (Schemes 3 and 4). Use of polymer-supported DCC gave higher yields and led to more facile isolation of the product. This resin contains 1% cross-linked poly(styrene-co-divinylbenzene) with a typical loading of 1.3 mmol/g of carbodiimide. The amino group of the amino acid was protected during the coupling reaction with either Fmoc or Cbz. Selected amino acid precursors (serine and tyrosine) also required benzyl protection of their phenolic groups (compounds **10d**, **11f** and **11g**). The required lysine precursor was available with both amino groups protected with the Fmoc group. Products were obtained in yields of over 44% with the exception of lysine-analogue **10c** (6%).

Initially the Fmoc-group was cleaved using the base piperidine [19, 20]. When 5% piperidine in dichloromethane [21] was used to remove the Fmoc-group from amino acid-β-lactam conjugates, difficulties in purification of the product arose. A second method using tetrabutylammonium fluoride (TBAF, 2 equivalents) and a thiol (1-octanethiol, 10 equivalents) to scavenge the liberated dibenzofulvene was investigated. This method

removes the Fmoc group from a variety of amino acid conjugates in up to 100% yield within 1 minute [22]. It was successfully applied to remove the Fmoc group from the β-lactam derivatives **10a-10d** and **11a - 11g** (Scheme 3), giving easily isolated products **10g - 10j** and **11i - 11o** in high yield, in less than 10 minutes as monitored by TLC. Cbz- and Bn-groups were removed by hydrogenolysis (H_2/PdC) from intermediates **10e**, **10f**, **10j**, **11h**, **11n** and **11o** to afford compounds **10k**, **10l**, **10m**, **11p**, **11q** and **11r** respectively (Scheme 4).

3. Solubility

Selected compounds **9**, **9a**, **10**, **10g**, **11** and **11l** were shown to be stable in stable in the pH range 4-9 and in human plasma, with half-lives greater than 24 h (data not shown). The solubility of the novel β-lactam derivatives was measured and compared to that of the parent compounds. The phosphate derivatives showed increased aqueous solubility compared to the insoluble parent compounds (Table 1). The phenolic β-lactams **7**, **8** and **9** did not display any detectable aqueous solubility, while phosphate derivatives **7a**, **8a** and **9a** were soluble at 0.3 mg/mL, 0.14 mg/mL and 0.45 mg/mL respectively in water, greater than the recommended minimum solubility of 0.05 mg/mL which is desirable for potential drug candidates [23]. The increase in solubility is reflective of the increase in hydrophilicity of the molecules, as indicated by their lower cLogP values (Table 1). All amino acid β-lactam amides assessed showed increased aqueous solubility compared to parent β-lactams **10** and **11** (Tables 2 and 3). The highest solubility was obtained with the alanine derivative **11j** (0.37 mg/mL), which again is favourable compared to the minimum desired value of 0.05 mg/mL. The observed increase in solubility due to the

phosphate or amino acid group improves the pharmacokinetic profile of the parent compound. Amide **7g** and amine **7l** were not useful as water soluble compounds.

4. Biochemical Results and Discussion

4.1. Antiproliferative Effects of Phosphate Ester and Amino Acid Amide β-Lactams

Stilbene **2** was insoluble in water and the development of **2a** represented the most successful prodrug of **2**; it retained the activity of the parent compound, had improved solubility and good stability [8]. Phosphate esters of three phenolic β-lactam compounds with potent IC_{50} values in antiproliferative assays (**7**, **8** and **9**) were prepared to investigate if improvements in aqueous solubility could be obtained without compromising antiproliferative activity. The antiproliferative activities of parent compounds were also previously demonstrated in a variety of cell types in the NCI cell line screen [4, 5]. The antiproliferative activity of all compounds was assessed using human breast carcinoma MCF-7 cells.

The β-lactam phosphate esters **7a**, **8a** and **9a** had IC_{50} values of 71 nM, 20 nM and 22 nM respectively and had slightly decreased potency compared to the corresponding parent compounds (Table 1). The IC_{50} values for the phosphate esters follow approximately the same trend as their parent compounds, as **8** and **9** are more potent than **7**, with **8a** and **9a** are more potent than **7a**.

The antiproliferative activity of β-lactam amino acid conjugates **10g**-**10i**, **10k**-**10m**, **11i**-**11m** and **11p**-**11r** was assessed (Tables 2 and 3). Derivatives of compound **10** were

extremely potent (Table 2), with alanine derivative **10h**, phenylalanine derivative **10k** and valine derivative **10l** showing activity greater than or equal to that of the parent amino compound **10** (IC_{50} values of 27 nM, 35 nM and 51 nM respectively compared to 51 nM for **10**). Valine derivative **11l** (IC_{50} value of 460 nM) was the most potent derivative of **11**. Alanine (**11j**), serine (**11q**) and tyrosine (**11r**) derivatives also had IC_{50} values in the nanomolar range comparable to that of the parent compound **11** (760 nM, 780 nM and 740 nM respectively, 650 nM for **11**).

4.2. Effects on Tubulin Polymerisation

Inhibition of tubulin polymerisation for existing phosphate prodrugs **1a**, **2a** and **4a** differ, with intact **1a** [6] and **2a** [24] displaying no intrinsic tubulin binding affinity while **4a** displays 40% of the activity of the parent compound **4** [14]. The effects of **5a** on *in vitro* tubulin polymerisation have not been reported, but it is known that amino acid amide derivatives of **6**, including **6a**, do not inhibit the polymerisation of tubulin at concentrations up to 40 μM [17]. It has previously been demonstrated that compounds **7**, **8** and **9** inhibit the polymerisation of tubulin [4, 5]. Representative β-lactams **7a** and **9a** (phosphate esters) and **10k** and **11l** (amino acid amides) were selected for *in vitro* tubulin polymerisation studies.

Phosphate β-lactam esters **7a** and **9a** did not inhibit the *in vitro* polymerisation of tubulin at a concentration of 10 μM (Figure 4). Given their potent antiproliferative effects in MCF-7 cells, this result indicates that bioconversion is necessary for the biochemical effects of the phosphate compounds. In contrast, the amino acid β-lactam conjugates **10k**

and **11l** inhibited *in vitro* polymerisation of tubulin in a concentration dependent manner (shown for **11l** in Figure 5), with IC_{50} values of 2.4 µM and 5.2 µM respectively. This indicates that bioconversion is not necessary for these compounds, although *in vivo* cleavage of the amide linkage is extremely likely to occur. For example, it has been reported that aminopeptidases on the surface of erythrocytes catalyse the cleavage of **5a** to **5** [21].

4.3. Immunofluorescence Studies

In addition to *in vitro* tubulin polymerisation studies, we investigated alterations in the microtubule network induced by β-lactams **8a, 9a, 10k** and **11l** in MCF-7 cell culture by confocal microscopy. Confocal analysis of MCF-7 cells stained with α-tubulin mAb demonstrated a well organised microtubular network in control cells (Figure 6, A+E and Figure 7, A+D). Exposure to any of the four β-lactam conjugates **8a**, **9a**, **10k** and **11l** for 16 h led to a complete loss of microtubule formation consistent with depolymerised microtubules (Figures 6 and 7). Additionally, cells treated with β-lactams **8a**, **9a**, **10k** and **11l** contained multiple micronuclei - a phenomenon described as mitotic catastrophe. Mitotic catastrophe is a type of programmed cell death in response to DNA damage, characterised by multinucleated cells [25]. We have previously noted mitotic catastrophe for related β-lactam derivatives [26]. The findings are in agreement with previously published studies, where **2** induced mitotic catastrophe in non-small cell lung cancer cells [27, 28] and **2a** in chronic lymphocytic leukaemia cells [29]. Mitotic catastrophe has also been demonstrated for **2** and related derivatives in both human endothelial cells (HUVEC), human lung carcinoma cells (H460) [30] and human breast cancer cells

(MCF-7) [26]. The confocal imaging results confirm that both the phosphate and amino acid β-lactam conjugates are targeting tubulin.

4.4. Analysis of DNA Content by Flow Cytometry

We next examined the effects of **2**, parent β-lactams **7**, **8** and **9** and β-lactam derivatives **7a**, **8a**, **9a**, **10k** and **11l** on the cell cycle of MCF-7 cells by flow cytometric analysis of propidium iodide stained cells (Figure 8). In this whole-cell assay, both phosphate and amino acid β-lactam prodrugs caused increases in the percentage of cells in the sub-G1 and G_2/M phases, indicative of apoptosis and G_2/M arrest respectively. There were no significant differences in the percentage of cells in sub-G1 and G_2/M phases between the parent β-lactams and the derivatives.

5. Molecular Modelling Studies

The tubulin binding and immunofluorescence studies clearly demonstrate that tubulin is the target of the new β-lactam derivatives. Based on structural similarities between **2** and the β-lactams reported in this study, we propose that they interact with the colchicine binding site of tubulin as demonstrated for **2** and structurally related, conformationally constrained analogues of **2** [30-32]. Molecular modelling studies were performed to investigate potential interactions for the intact β-lactam amino acid amides **10k** and **11l**, considering that they inhibited tubulin polymerisation without chemical or enzymatic modification. The reported X-ray structure of tubulin cocrystallized with a colchicine derivative, *N*-deacetyl-*N*-(2-mercaptoacetyl)colchicine (DAMA-colchicine, PDB entry 1SA0) was used for the docking study [33]. β-Lactams were isolated as racemic mixtures and the presence of two enantiomers has been demonstrated [4]. Molecular modelling studies were carried out with both enantiomers and results of the best fitting *3S*,4R** enantiomer are illustrated. The β-lactam phosphate esters **7a**, **8a** and **9a** had no effect in the *in vitro* tubulin polymerisation assay, indicating that hydrolysis is required for their effects, and therefore no molecular modelling was investigated for those compounds.

Molecular docking studies of parent compounds **10** and **11** (Figures 9A and 10A) predicted similar interactions to those previously reported to be of importance for binding at the colchicine site, including important interactions with Cys241 and Val318 [33, 34]. Additionally, there is direct bond between compound **11** and the Met259 residue. Both compounds are orientated in a similar manner to DAMA-colchicine, with the 3,4,5-

trimethoxyaryl A ring and 4-methoxyaryl B ring occupying the same position in the binding pocket (Figures S1 and S3, Supplementary Information).

Comparison of the docked structures of **10** and **10k** indicated that the core β-lactam, A and B rings are orientated in the same way in the binding pocket (Figure 9A and B, and Figures S1 and S2, Supplementary Information). All but one of the 24 binding contacts for **10** are also predicted for **10k**, the exception being an interaction with Asn249. The phenylalanine substituent on ring C extends further and makes numerous additional contacts, for example with Lys254 and Lys352 (Figure 9B). We have previously shown that bulky substituents directly attached to ring C are detrimental to activity [4]. In this series of β-lactams the potency of **10k** in MCF-7 cells indicates that the linear chain linking the two aromatic rings (ring C to the aromatic ring of phenylalanine) is essential to maintain potent *in vitro* activity.

The docked structures of **11** and **11l** are illustrated in Figure 10. Additional binding interactions associated with the valine moiety are predicted for **11l**, namely with Gln11, Gly143, Ser178, Glu183, Tyr224 and Gln247. The predicted docking position for both the core β-lactam and A rings is similar for these two structures. However, the positioning of the B and C rings of both compounds is different (Figures S3 and S4, Supplementary Information). We have previously noted this 'flip' in relative orientation of the B and C rings for β-lactams when larger substitutents are present on ring B [26]. The orientation predicted for **11l** is consistent with our previous experience that bulky substitution to the B ring forces a change in molecular orientation within the colchicine

binding site. This is not necessarily associated with a decrease in antiproliferative activity, as **11l** is more potent in MCF-7 cells than **11**. However, we must once again emphasize that hydrolysis to yield the parent amine compounds **10** and **11** is predicted to occur *in vivo* prior to interaction with tubulin. These *in silico* studies provide useful information for the development of future antiproliferative agents that interact with the colchicine binding site of tubulin, possibly exploiting the additional binding interactions predicted for **10k** and **11l**.

6. Discussion

Our previously reported β-lactams are amongst the most potent analogues of **2** known, with selected compounds showing sub-nanomolar antiproliferative activity (e.g. IC_{50} for **9** in MCF-7 cells is 0.8 nM). However, these compounds are not sufficiently water soluble for further development and a prodrug strategy was devised to improve this physiochemical characteristic. Potentially useful prodrugs were synthesised including three β-lactam phosphate esters, two β-lactam esters and eighteen amino acid amides. A number of novel compounds exhibited greatly improved aqueous solubility compared to the parent compounds.

The primary *in vitro* degradation pathway of concern for phosphates is hydrolysis, and the electronic environment and substitution on the prodrug moiety may have profound effects on stability [35]. Two previously reported prodrugs of **2** were found to be stable in aqueous solution [36]. When the stability of phosphate ester prodrugs **2a** and **3a** was evaluated in murine plasma, there was little evidence of dephosphorylation even after 3

hours. In contrast, **2a** and **3a** were rapidly dephosphorylated in tumor (MAC29) and liver preparations [37]. It is expected that rapid *in vivo* dephosphorylation would occur for the β-lactam phosphates **7a**, **8a** and **9a** in a manner similar to that documented for **2a** [12]. *In vitro*, a selection of representative β-lactam phosphate esters were demonstrated to be stable in the pH range 4-9 and in plasma, with half-lives greater than 24 hours. The β-lactam amino acid amides are also stable at three pH values (Table 4). These amides are expected to be hydrolysed by peptidases *in vivo* in a similar fashion to **5a** [21]. Further detailed stability studies on the β-lactam phosphate esters and amino acid amides are ongoing.

Biochemical profiling of the β-lactam derivatives confirmed their antiproliferative and tubulin-targeting effects. For both series of compounds, antiproliferative activity was noted in the nanomolar concentration range, with **8a**, **9a**, **7a** and **10k** having the lowest IC_{50} values (20, 22, 71 and 27 nM respectively). Immunofluorescence microscopy studies showed disruption of the microtubule network and mitotic catastrophe for both the phosphate and amino acid series of analogues. In cell-cycle analysis the percentage of cells in sub-G_1 (indicative of cell death) was significantly increased compared to the control.

In the antiproliferative, immunofluorescence and cell cycle analysis assays, whole cells are used and it is expected that bioconversion of the β-lactam derivatives to the parent compounds is possible. Both series of compounds had the same potent biochemical effects in these assays. However, we noted a significant difference between the phosphate

and amino acid analogues in the tubulin polymerisation assay, which uses isolated, purified tubulin and in which metabolic enzymes are not present. In this assay, the phosphate prodrugs did not inhibit tubulin polymerisation. The amino acid β-lactam conjugates, taking **10k** and **11l** as representative examples, inhibited tubulin polymerisation *in vitro* in the absence of enzymatic action. These results indicate that the phosphate derivatives are true prodrugs requiring enzymatic activation, while the intact amino acid derivatives are microtubule poisons in their own right. However, due to the widespread presence of aminopeptidases in the blood, it is likely that they may be hydrolysed in vivo before reaching their target site of action. Regardless of this, we have demonstrated they can act as microtubule depolymerisers in either form.

7. Conclusion

We describe the synthesis and biochemical effects of novel series of antiproliferative phosphate ester and amino acid amide β-lactam derivatives, designed to overcome the pharmacokinetic hurdle of poor aqueous solubility observed for previously reported compounds [4]. The novel compounds displayed increased aqueous solubility, which is an advantage for drug delivery. The strategic modification of previously described β-lactam CA-4 derivatives by inclusion of phosphate or amino acid moieties did not adversely influence their antiproliferative and tubulin targeting properties, as determined by antiproliferative assays, cell cycle assays and analysis of the microtubular network by confocal microscopy. Novel derivatives had nanomolar antiproliferative IC_{50} values in MCF-7 cells and disrupted the microtubule network. The potent activity and microtubule disrupting effects of the β-lactam derivatives warrants further investigations.

8. Experimental Methods

All reagents were commercially available and were used without further purification unless otherwise indicated. IR spectra were recorded as thin films on NaCl plates or as KBr discs on a Perkin-Elmer Paragon 100 FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker Avance DPX 400 instrument at 20^{o}C, 400.13MHz for ^{1}H spectra, 100.61MHz for ^{13}C spectra, in $CDCl_3$, CD_3OD or DMSO-d_6 (internal standard tetramethylsilane) by Dr. John O'Brien and Dr. Manuel Ruether in the School of Chemistry, Trinity College Dublin. High resolution accurate mass determinations (HRMS) for all final target compounds were obtained on a Micromass Time of Flight mass spectrometer equipped with electrospray ionisation (ES) interface operated in the positive ion mode at the High Resolution Mass Spectrometry Laboratory by Dr. Martin Feeney in the School of Chemistry, Trinity College Dublin. Thin layer chromatography was performed using Merck Silica gel 60 TLC aluminium sheets with fluorescent indicator visualizing with UV light at 254 nm. Flash chromatography was carried out using standard silica gel 60 (230-400 mesh) obtained from Merck. Analytical high-performance liquid chromatography (HPLC) to determine the purity of the final compounds was performed using a Waters 2487 Dual Wavelength Absorbance detector, a Waters 1525 binary HPLC pump, a Waters In-Line Degasser AF and a Waters 717plus Autosampler. The column used was a Varian Pursuit XRs C18 reverse phase 150 x 4.6 mm chromatography column. Samples were detected using a wavelength of 254 nm. All samples were analysed using acetonitrile (70%): water (30%) over 10 min and a flow rate of 1 mL/min. The purity of the tested compounds was $\geq$95%. Polymer-supported DCC was obtained from (Biotage®). Syntheses of imine **12c** [18] and β-lactams **7**, **8**, **9**, **10** and

11 [4, 5] were achieved as previously reported. Experimental characterisation of compounds **8a**, **8b**, **9a**, **9b**, **10a-10d**, **10f-10j**, **10l**, **10m**, **11a-11c**, **11e-11k**, **11m-11r** is contained in the supplementary information.

8.1 Chemical Synthesis

8.1.1. General procedure for synthesis of imines

The appropriate amine (10 mmol) was refluxed with the appropriate aldehyde (10 mmol) in ethanol (50 mL) for 3 h. The reaction mixture was reduced *in vacuo*, and the resulting solution was allowed to stand until solid product crystallized from solution. The resulting imine was recrystallized from ethanol.

8.1.1.1. 2-Methoxy-5-(((3,4,5-trimethoxyphenyl)imino)methyl)phenyl acetate (12a) was obtained from 3,4,5-trimethoxyaniline and 5-formyl-2-methoxyphenyl acetate as a white powder (64% yield); mp 120 °C; IR (KBr disk) υ_{max}: 1608 cm^{-1} (C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.22 (s, 3H, CH_3), 3.85 (m, 12H, OCH_3), 6.51 (s, 2H, ArH), 7.08 (d, 1H, J=4.5 Hz, ArH), 7.28 (s, 1H, ArH), 7.79 (m, 1H, ArH), 8.39 (s, 1H, C=N); HRMS: calculated for $C_{19}H_{22}NO_6$: 360.1449; found 360.1430 $[M+H]^+$.

8.1.1.2. 2-Methoxy-5-(((3,4,5-trimethoxyphenyl)imino)methyl)phenyl benzoate (12b) was obtained from 3,4,5-trimethoxyaniline and 5-formyl-2-methoxyphenyl benzoate as peach coloured crystals (75% yield); mp 120 °C; IR (KBr disk) υ_{max}: 1611 cm^{-1} (C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.90 (m, 12H, OCH_3), 7.11 (s, 1H, ArH), 7.28 (s, 2H, ArH), 7.55 (m, 2H, ArH), 7.67 (m,1H, ArH), 7.83 (m, 2H, ArH), 8.24 (d, 2H, J=1 Hz, ArH), 8.43 (s, 1H, C=N); HRMS: calculated for $C_{24}H_{24}NO_6$: 422.1604; found 422.1582$[M+H]^+$.

8.1.2. General procedure for synthesis of 7b, 8b and 9b

Carbon tetrachloride (85 mmol) was added to a solution of appropriate phenol (17 mmol) in acetonitrile (100 mL) at 0 °C. The resulting solution was stirred for 10 minutes prior to addition of diisopropylethylamine (35 mmol) and DMAP (1.7 mmol). Then, dibenzyl phosphite (24.5 mmol) was added dropwise. When the reaction was complete as indicated by TLC, KH_2PO_4 (aq., 0.5 M) was added and the mixture was allowed to warm to room temperature. An ethyl acetate extract (3 × 50 mL) was washed with saturated NaCl (100 mL) followed by water (100 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure and the product isolated by flash column chromatography (hexane: ethyl acetate gradient, 88:12 to 50:50).

8.1.2.1. Dibenzyl (2-methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl) phosphate (7b) was prepared according to the general procedure above from 4-(3-hydroxy-4-methoxyphenyl)-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (**7**) in 74.3% yield as a yellow oil; IR (NaCl film) ν_{max}: 1750 cm^{-1} (β-lactam -C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 3.73 (s, 6H, 2 × OCH_3), 3.78 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.25 (d, 1H, J=2 Hz, H_3), 4.78 (d, 1H, J=2 Hz, H_4), 5.15-5.18 (m, 4H, CH_2), 6.60 (s, 2H, ArH), 6.98 (d, 1H, J=8 Hz, ArH), 7.20 (m, 2H, ArH), 7.33-7.41 (m, 15H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 55.6 (OCH_3), 55.6 (OCH_3), 60.5 (OCH_3), 62.9 (C_3), 64.5 (C_4), 69.5 (OCH_2), 69.6 (OCH_2), 94.3 (CH, Ar), 112.9 (CH, Ar), 119.3 (CH, Ar), 122.8 (CH, Ar), 127.0 (CH, Ar), 127.4 (CH, Ar), 127.5 (CH, Ar), 127.6 (CH, Ar), 128.2 (CH, Ar), 128.2 (CH, Ar), 128.6 (CH, Ar), 133.1 (C, Ar), 134.0 (C, Ar), 153.1 (C, Ar), 165.0 (C=O); HRMS: calculated for $C_{39}H_{38}NO_9PNa$: 718.2182; found 718.2172 $[M+Na]^+$.

8.1.3. General method for synthesis of phosphates 7a, 8a and 9a

β-Lactams **7b**, **8b** or **9b** (2 mmol) were dissolved in ethanol: ethyl acetate (50 mL; 1:1 mixture) and hydrogenated over 10% Pd/C (1.2 g) until complete on TLC; typically less than 3 hours. The catalyst was removed by filtration through Celite, the solvent was evaporated under reduced pressure and the product was isolated by flash column chromatography (hexane: ethyl acetate gradient, 88:12 to 50:50).

8.1.3.1. 2-Methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl dihydrogen phosphate (7a) was prepared from **7b** as described in the general method above. The product was obtained as a brown oil in 93% yield; IR (KBr disk) ν_{max}: 1747 cm^{-1} (β-lactam -C=O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.59 (s, 3H, OCH_3), 3.67 (s, 6H, 2 × OCH_3), 3.77 (s, 3H, OCH_3), 4.43 (d, 1H, J=2.5 Hz, H_3), 5.24 (d, 1H, J=2.5 Hz, H_4), 6.63 (s, 2H, ArH), 7.09 (d, 1H, ArH, J=8.5 Hz), 7.35 (m, 1H, ArH), 7.36-7.42 (m, 5H, ArH), 7.51 (s, 1H, ArH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 56.1 (OCH_3), 56.2 (OCH_3), 60.5 (OCH_3), 62.2 (C_3), 64.0 (C_4), 95.5 (CH, Ar), 113.7 (CH, Ar), 119.6 (CH, Ar), 122.9 (CH, Ar), 127.9 (CH, Ar), 128.0 (CH, Ar), 128.2 (CH, Ar), 128.7 (CH, Ar), 129.4 (CH, Ar), 129.7 (CH, Ar), 133.5 (C, Ar), 134.3 (C, Ar), 135.2 (C, Ar), 150.9 (C, Ar), 151.0 (C, Ar), 153.6 (C, Ar), 165.8 (C=O); HRMS: Calculated for $C_{25}H_{26}NNaO_9PS$: 538.1243; found 538.1252 $[M+Na]^+$.

8.1.4. 2-Methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl 2-(((benzyloxy)carbonyl)amino)propanoate (7e). N-Benzyloxycarbonyl-L-alanine

(0.23 mmol), DCC (0.23 mmol) and DMAP (0.23 mmol) were stirred in anhydrous DCM (5 mL) at 0 ºC. A solution of the β-lactam **7** (0.23 mmol) in anhydrous DCM (5 mL) was added dropwise over five min. The reaction was stirred for 16 h under a nitrogen atmosphere at ambient temperature. Reaction was monitored via TLC. After 24 hours, dichloromethane (50 mL) was added and the mixture was filtered. The solvent was removed by washing with water (5 × 50 mL). The organic solvent containing the product was evaporated under reduced pressure. The product was isolated by flash column chromatography (dichloromethane: methanol gradient), to afford the product **7e** (88%). IR (NaCl film) ν_{max}: 1752.9 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.62 (3H, d, J=7 Hz, CH_3), 3.75-3.92 (m, 12H, 4 × OCH_3), 4.30-4.32 (m, 1H, H_3), 4.69 (1H, q, J=7 Hz, CH), 4.83-4.87 (m, 0.5H, CH, H_4), 5.15-5.44 (m, 0.5H, CH, H_4), 5.39-5.44 (2H, m, OCH_2), 6.61-6.64 (2H, m, ArH), 6.88-6.95 (m, 1H, ArH), 7.01-7.03 (m, 1H, ArH), 7.16-7.17 (m, 1H, ArH), 7.29-7.41 (m, 10H, ArH).^{13}C NMR (100 MHz, $CDCl_3$) δ 18.7 (CH_3), 18.8 (CH_3), 49.7 (C), 56.1 (OCH_3), 60.9 (OCH_3), 63.4 (C_3), 63.8 (C_3), 64.9 (C_4), 65.0 (CH_2), 67.1 (CH_2), 94.8 (CH, Ar), 94.9 (CH, Ar), 111.1 (CH, Ar), 112.0 (CH, Ar), 113.1 (CH, Ar), 113.1 (CH, Ar), 117.8 (CH, Ar), 120.8 (CH, Ar), 124.6 (CH, Ar), 124.6 (CH, Ar), 127.5 (CH, Ar), 127.9 (CH, Ar), 128.0 (CH, Ar), 128.1 (CH, Ar), 128.2 (CH, Ar), 128.6 (CH, Ar), 129.0 (CH, Ar), 129.1 (CH, Ar), 129.9 (CH, Ar), 130.5 (CH, Ar), 133.6 (C, Ar), 133.7 (C, Ar), 134.5 (C, Ar), 134.5 (C, Ar), 134.8 (C, Ar), 136.2 (C, Ar), 139.9 (C, Ar), 146.4 (C, Ar), 146.9 (C, Ar), 151.2 (C, Ar), 153.5 (C, Ar), 153.6 (C, Ar), 155.6 (C, Ar), 165.5 (C=O), 165.6 (C=O), 171.0 (C=O), 171.1 (C=O); HRMS: Calculated for $C_{36}H_{36}N_2O_9Na$: 663.2319; Found 663.2332 $[M+Na]^+$.

8.1.5. 2-Methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl 2-(((benzyloxy)carbonyl)amino)-3-phenylpropanoate (7f). N-Benzyloxycarbonyl-L-phenylalanine (0.23 mmol), DCC (0.23 mmol) and DMAP (0.23 mmol) were stirred in anhydrous DCM (5 mL) at 0 ºC. A solution of **7** (0.23 mmol) in anhydrous DCM (5 mL) was added dropwise over five min. Following the method described above for the preparation of **7e**, the product **7f** was isolated (79%). IR (NaCl film) ν_{max}: 1754.7 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.24-3.38 (m, 2H, CH_2), 3.75-3.86 (m, 12H, 4 × OCH_3), 4.32 (m, 1H, H_3), 4.87 (m, 1H, H_4), 4.95-4.99 (m, 1H, CH), 5.13 (s, 2H, CH_2), 6.63 (s, 2H, ArH), 7.00-7.04 (m, 2H, ArH), 7.29-7.42 (m, 16H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 37.6 (CH_2), 37.7 (CH_2), 54.3 (C), 55.5 (OCH_3), 55.6 (OCH_3), 60.5 (OCH_3), 62.9 (C_3), 64.5 (C_4), 66.6 (OCH_2), 94.3 (CH, Ar), 112.6 (CH, Ar), 112.7 (CH, Ar), 120.4 (CH, Ar), 124.2 (CH, Ar), 126.8 (CH, Ar), 127.0 (CH, Ar), 127.6 (CH, Ar), 127.8 (CH, Ar), 128.1 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.7 (CH, Ar), 128.8 (CH, Ar), 129.0 (CH, Ar), 129.1 (CH, Ar), 129.4 (CH, Ar), 129.5 (CH, Ar), 133.1 (C, Ar), 134.0 (C, Ar), 134.1 (C, Ar), 135.0 (C, Ar), 135.1 (C, Ar), 139.3 (C, Ar), 150.9 (C, Ar), 153.1 (C, Ar), 155.2 (C, Ar), 164.9 (C=O), 165.0 (C=O), 169.1 (C=O), 169.2 (C=O); HRMS: Calculated for $C_{42}H_{41}N_2O_9$: 717.2812; found 717.2813 $[M+H]^+$.

8.1.6. Acetylamino-acetic acid 2-methoxy-5-[4-oxo-3-phenyl-1-(3,4,5-trimethoxy-phenyl)azetidin-2-yl]phenyl ester (7g). N-Acetylglycine (0.23 mmol), DCC (0.23 mmol) and DMAP (0.23 mmol) were stirred in anhydrous DCM (5 mL) at 0 ºC. A solution of **7** (0.23 mmol) in anhydrous DCM (5 mL) was added dropwise over five min. The reaction was stirred for 16 h under a nitrogen atmosphere at ambient temperature.

Reaction was monitored via TLC. After 24 hours, dichloromethane (50 mL) was added and the mixture was filtered. The solvent was removed by washing with water (5 × 50 mL). The organic solvent containing the product was evaporated under reduced pressure. The product was isolated by flash column chromatography (dichloromethane: methanol gradient), to afford the product (72%). IR (NaCl film) ν_{max}: 1753.3 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.07 (s, 3H, CH_3), 3.73 (s, 6H, 2 × OCH_3), 3.79 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 4.29 (d, 1H, J=2.5 Hz, H_3), 4.32-4.33 (m, 2H, CH_2), 4.86 (d, 1H, J=2 Hz, H_4), 6.60 (s, 2H, ArH), 7.02 (d, 1H, J=8.5 Hz, ArH), 7.15 (m, 1H, ArH), 7.31-7.41 (m, 6H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 22.5 (CH_3), 40.7 (CH_2), 55.6 (OCH_3), 60.5 (OCH_3), 62.9 (C_3), 64.5 (C_4), 94.3 (CH, Ar), 112.7 (CH, Ar), 120.2 (CH, Ar), 124.3 (CH, Ar), 126.9 (CH, Ar), 127.6 (CH, Ar), 128.6 (CH, Ar), 129.5 (CH, Ar), 133.1 (C, Ar), 133.9 (C, Ar), 134.0 (C, Ar), 139.3 (C, Ar), 150.8 (C, Ar), 153.1 (C, Ar), 165.0 (C=O), 167.7 (C=O), 169.9 (C=O); HRMS: Calculated for $C_{29}H_{30}NO_8Na$: 557.1900; found 557.1917 $[M+Na]^+$.

8.1.7. 2-Methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl acetate (7i). The imine **12a** (5mmol) and triethyl amine (15 mmol) were added to anhydrous dichloromethane (50 mL) and the mixture heated to reflux under nitrogen atmosphere. Acetyl chloride (7.5 mmol) was injected dropwise through a septum, and the mixture was allowed to reflux for 3 h. The reaction mixture was then cooled, washed with water, (2 × 50 mL) and sodium bicarbonate solution (aq., 50 mL). The organic layer was dried with anhydrous Na_2SO_4, and the solvent evaporated to dryness *in vacuo*. The residue was purified by flash chromatography (hexane ethyl acetate, 50:50) to afford the

β-lactam product as colourless crystals from methanol (7%), Mp 70 ^{o}C. IR (KBr disk) ν_{max}: 1725.1 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.12 (3H, s, CH_3), 3.75 (6H, s, 2 × OCH_3), 3.80 (3H, s, OCH_3), 3.87 (3H, s, 2 × OCH_3), 4.32 (1H, d, J=2.5 Hz, H_3), 4.86 (1H, d, J=2.5 Hz, H_4), 6.64 (2H, s, ArH), 7.02 (1H, m, ArH), 7.13(1H, d, J=2 Hz, ArH), 7.27-7.40 (6H, m, ArH); HRMS: Calculated for $C_{27}H_{27}NO_7Na$: 500.1685; found: 500.1688 $[M+Na]^+$.

8.1.8. 2-Methoxy-5--4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl benzoate (7j). The imine **12b** (5mmol) and triethyl amine (15 mmol) were added to anhydrous dichloromethane (50 mL) and the mixture heated to reflux under nitrogen atmosphere. Phenylacetyl chloride (7.5 mmol) was injected dropwise through a septum, and the mixture was allowed to reflux for 3 h, following the method described above for the preparation of **7i**. The β-lactam product **7j** was obtained as colourless crystals from methanol (8%), Mp 73 ^{o}C. IR (NaCl film) ν_{max}: 1752.0 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.84 (s, 6H, 2 × OCH_3), 3.84 (s, 3H, OCH_3), (s, 3H, OCH_3), 4.36 (d, 1H, J=2.9 Hz, H_3), 4.90 (d, 1H, J=2.9 Hz, H_4), 6.63 (s, 2H, ArH), 7.07-7.31 (m, 2H, ArH), 7.35-7.39 (m, 4H, ArH), 7.52-7.83 (m, 4H, ArH), 8.20-8.24, (m, 3H, ArH); HRMS: Calculated for $C_{32}H_{29}NO_7Na$: 562.1842; found: 562.1841 $[M+Na]^+$.

8.1.9. [3-(2-Bromoethoxy)-4-methoxybenzylidene]-(3,4,5-trimethoxyphenyl)amine (12d). Imine **12c** (5 mmol) and tertabutylammonium sulfate (4.5 mmol) were dissolved in dibromoethane (50 mmol). Sodium hydroxide solution (1 M, 40 mL) was added to the reaction mixture. The reaction was vigorously stirred for 16 h. Upon completion, the

reaction mixture was diluted with dichloromethane (100 mL) and saturated sodium hydrogen carbonate (100 mL). The organic phase was separated, while the aqueous phase was extracted with dichloromethane (2 × 100 mL). The organic phases were combined, dried over Mg_2SO_4 and evaporated to dryness *in vacuo*. The resulting crystals were recrystallised from ethanol to afford the product (83%); IR (KBr disk) ν_{max}: 1622.8 (C=N); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.89-3.97 (m, 12H, 3 × OCH_3), 3.74 (t, 2H, J=6.5 Hz, CH_2Br), 4.46 (t, 2H, CH_2O), 6.51 (s, 2H, ArH), 6.99 (d, 1H, J=8.5 Hz, ArH), 7.39 (d, 1H, J=8 Hz, ArH), 7.62 (s, 1H, ArH), 8.40 (s, 1H, HC=N); ^{13}C NMR (100 MHz, $CDCl_3$) δ 29.1 (CH_2Br), 55.6 (OCH_3), 60.6 (OCH_3), 68.4 (CH_2O), 97.6 (CH, Ar), 110.8 (CH, Ar), 111.2 (CH, Ar), 124.8 (CH, Ar), 128.8 (CH, Ar), 135.7 (C, Ar), 143.0 (C, Ar), 147.5 (C, Ar), 153.1 (C, Ar), 158.4 (C=N); HRMS: Calculated for $C_{19}H_{23}NO_5Br$: 424.0760; found 424.075 $[M+H]^+$.

8.1.10. 4-[3-(2-Bromoethoxy)-4-methoxyphenyl]-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (7k). Imine **12d** (2.0 mmol) and triethylamine (2.6 mmol) were dissolved in anhydrous dichloromethane (10 mL). A solution of phenylacetyl chloride (2.6 mmol) in anhydrous dichloromethane (5 mL) was added slowly via syringe. The reaction was refluxed for two hours, then allowed cool and evaporated to dryness. The residue was purified by flash chromatography (hexane: ethyl acetate, 1:1) to afford **7k** as a resin (29%); IR (NaCl film) ν_{max} 1749.8 (β-lactam C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.64 (t, 2H, CH_2Br, J=6.5 Hz), 3.75 (s, 6H, OCH_3), 3.80 (s, 3H, OCH_3), 3.91 (s, 3H, OCH_3), 4.29-4.34 (m, 3H, OCH_2, H_3), 4.86 (d, 1H, J=2.5 Hz), 6.63 (s, 2H), 6.93-6.96 (m, 2H), 7.05-7.08 (m, 1H), 7.34-7.40 (m, 5H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.4

(CH_2Br), 55.6 (OCH_3), 59.9 (OCH_3), 60.5 (OCH_3), 63.4 (C_3), 64.5 (C_4), 68.9 (OCH_2), 94.4 (CH, Ar), 111.7 (CH, Ar), 112.1 (CH, Ar), 119.7 (CH, Ar), 126.9 (CH, Ar), 127.5 (CH, Ar), 128.6 (CH, Ar), 129.4 (CH, Ar), 130.5 (CH, Ar), 133.2 (C, Ar), 134.2 (C, Ar), 147.7 (C, Ar), 149.8 (C, Ar), 153.1 (C, Ar), 165.1 (C=O); HRMS: Calculated for $C_{27}H_{28}NO_6NaBr$: 564.0998, found 564.1005 $[M+Na]^+$.

8.1.11. 4-[4-Methoxy-3-(2-methylaminoethoxy)phenyl]-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-one (7l). β-Lactam **7k** (0.2 mmol) and methylamine 2.0 M solution (4.0 mmol) were dissolved in anhydrous tetrahydrofuran (10 mL) in a high pressure tube. The sealed tube was heated to 60 ºC for 6 h. The reaction mixture was allowed cool and sodium carbonate/sodium hydrogen carbonate pH 10 buffer solution was added (25 mL). The reaction mixture was extracted with dichloromethane (3 × 50 mL). The combined organic phases were dried over Mg_2SO_4 and evaporated to dryness *in vacuo*. The residue was purified using flash chromatography (hexane: ethyl acetate, 1:1) to afford **7l** as a resin (30%); IR (NaCl film) ν_{max} 1746.6 (β-lactam C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.70 (s, 3H, NCH_3), 3.24 (m, 2H, CH_2), 3.75 (s, 6H, OCH_3), 3.79 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 4.22-4.30 (m, 3H, CH, OCH_2), 4,57 (broad s, 1H, NH), 4.86 (d, 1H, CH, J=2 Hz), 6.62 (s, 2H, ArH), 6.93-6.96 (m, 2H), 7.05-7.08 (m, 1H), 7.33-7.41 (m, 5H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 34.0 (NCH_3), 48.6 (CH_2), 55.6 (OCH_3), 55.9 (OCH_3), 60.5 (OCH_3), 63.3 (C_3), 64.5 (C_4), 66.0 (OCH_2), 94.4 (CH, Ar), 102.9 (CH, Ar), 111.5 (CH, Ar), 111.7 (CH, Ar), 119.7 (CH, Ar), 127.0 (CH, Ar), 127.5 (CH, Ar), 127.8 (CH, Ar), 127.9 (CH, Ar), 128.5 (CH, Ar), 128.6 (CH, Ar), 129.7 (CH, Ar), 130.5

(CH, Ar), 133.2 (C, Ar), 134.1 (C, Ar), 147.7 (C, Ar), 149.5 (C, Ar), 153.1 (C, Ar), 165.2 (C=O); HRMS: Calculated for $C_{28}H_{33}N_2O_6$: 493.2334; found 493.2337 $[M+H]^+$.

8.1.12. General method for synthesis of amides 10a-10f and 11a-11h

To a stirred solution of the appropriate amino β-lactam **10** or **11** (4.76 mmol) in anhydrous DMF (30 mL) were added DCC (5.7 mmol), Cbz- or Fmoc-protected L-amino acid (5.7 mmol) and HOBt.H_2O (7.3 mmol) at room temperature. After 24 hours, ethyl acetate (50 mL) was added and the mixture was filtered. DMF was removed by washing with water (5 by 50 mL). The organic solvent containing the product was evaporated under reduced pressure. The product was isolated by flash column chromatography (dichlormethane: methanol gradient).

8.1.12.1. Benzyl (1-((4-(2-(4-methoxyphenyl)-4-oxo-1-(3,4,5-trimethoxyphenyl)azetidin-3-yl)phenyl)amino)-1-oxo-3-phenylpropan-2-yl)carbamate (10e) was prepared from **10** and Cbz-protected phenylalanine following the general method above and was isolated as a yellow gel in 28% yield; IR (NaCl film) ν_{max}: 1743 cm^{-1} (β-lactam -C=O), 1689 cm^{-1} (-C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 3.11-3.25 (m, 2H, CH_2), 3.74 (s, 6H, 2 × OCH_3), 3.80 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 4.01 (m, 2H, CH_2), 4.24 (d, 1H, CH), 4.55 (d, 1H, CH), 4.82 (d, 1H, J=2.5 Hz, H_4), 5.49 (s, 1H), 6.61 (s, 2H, ArH), 6.96 (d, 2H, J=9.0 Hz, ArH), 7.25-7.35 (m, 18H, ArH), 7.67 (s, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 38.4 (CH_2), 53.0 (CH), 54.9 (OCH_3), 55.6 (OCH_3), 60.5 (OCH_3), 63.5 (C_3), 64.1 (C_4), 66.6 (OCH_2), 66.9 (OCH_2), 94.4 (CH, Ar), 114.2 (CH, Ar), 120.2 (CH, Ar), 125.4 (CH, Ar), 126.8 (CH, Ar), 127.6 (CH, Ar), 127.7

(CH, Ar), 127.9 (CH, Ar), 128.1 (CH, Ar), 128.5 (CH, Ar), 128.7 (CH, Ar), 128.8 (CH, Ar), 133.2 (C, Ar), 134.0 (C, Ar), 135.4 (C, Ar), 135.7 (C, Ar), 153.0 (C, Ar), 159.5 (C, Ar), 165.2 (C=O), 168.6 (C=O); HRMS: Calculated for $C_{42}H_{41}N_3O_8Na$: 738.2791; found 738.2777 $[M+Na]^+$.

8.1.12.2. (9H-Fluoren-9-yl)methyl (1-((2-methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl)amino)-3-methyl-1-oxobutan-2-yl)carbamate (11d) was prepared from **11** and Fmoc-protected valine following the general method above and was isolated as a brown oil in 51% yield; IR (NaCl film) ν_{max}: 1748 cm^{-1} (β-lactam -C=O), 1686 cm^{-1} (C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 1.01-1.04 (m, 6H, 2 × CH_3), 2.20-2.28 (m, 1H, CH), 3.75-3.88 (m, 12H, 4 × OCH_3), 4.18 (m, 2H), 4.24 (m, 1H, CH), 4.43-4.48 (m, 2H), 4.92 (s, 1H, CH), 5.49 (d, 1H, J=8.6 Hz), 6.66 (s, 2H, ArH), 6.81 (s, 1H, ArH), 6.92-6.94 (m, 1H, ArH), 7.16-7.18 (m, 1H, ArH), 7.31-7.45 (m, 9H, ArH), 7.62-7.63 (m, 2H, ArH), 7.77 (m, 2H, ArH), 8.23 (s, 1H, ArH), 8.54 (m, 1H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.5 (CH_3), 18.8 (CH_3), 30.7 (CH), 46.7 (CH), 55.1 (OCH_3), 55.5 (OCH_3), 55.6 (OCH_3), 55.7 (OCH_3), 60.5 (OCH_3), 61.0 (OCH_3), 63.3 (C_3), 63.6 (C_3), 64.3 (C_4), 64.4 (C_4), 66.8 (CH_2), 94.4 (CH, Ar), 94.4 (CH, Ar), 94.5 (CH, Ar), 110.1 (CH, Ar), 110.3 (CH, Ar), 111.3 (CH, Ar), 111.4 (CH, Ar), 115.8 (CH, Ar), 116.3 (CH, Ar), 117.9 (CH, Ar), 119.6 (CH, Ar), 120.8 (CH, Ar), 120.9 (CH, Ar), 124.6 (CH, Ar), 126.2 (CH, Ar), 126.6 (CH, Ar), 126.8 (CH, Ar), 127.0 (CH, Ar), 127.1 (CH, Ar), 127.2 (CH, Ar), 127.3 (CH, Ar), 127.4 (CH, Ar), 128.5 (CH, Ar), 128.6 (CH, Ar), 129.4 (CH, Ar), 133.2 (C, Ar), 133.4 (C, Ar), 133.9 (C, Ar), 134.1 (C, Ar), 134.4 (C, Ar), 136.1 (C, Ar), 140.8 (C, Ar), 143.2 (C, Ar), 143.3 (C, Ar), 147.8 (C, Ar), 153.0 (C, Ar), 156.0 (C,

Ar), 165.3 (C=O), 172.1 (C=O); HRMS: Calculated for $C_{45}H_{45}N_3O_8Na$: 778.3104; found 778.3107 $[M+Na]^+$.

8.1.13. General methods for removal of Fmoc protecting group for preparation of 10g-10j and 11i-11o

General method 1: To the Fmoc-amino acid amide (2.1 mmol) at room temperature in $CHCl_3$ (55 mL) was added piperidine (2 mL). The mixture was stirred and monitored until complete on TLC. The solvent was evaporated under reduced pressure. The product was isolated by flash column chromatography (dichloromethane: methanol gradient, 100:0 to 90:10).

General method 2: To the Fmoc-amino acid amide dissolved in CH_2Cl_2 (minimum volume, 1-2 mL) was added 1-octanethiol (10 equiv.) and TBAF (2 equiv.). The mixture was stirred at room temperature until complete on TLC, typically within 10 minutes. The solvent was evaporated under reduced pressure and product was isolated by flash column chromatography (dichloromethane: methanol gradient, 100:0 to 90:10).

8.1.13.1. 2-Amino-N-(2-methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl)-3-methylbutanamide (11l) was prepared from **11d** by general method 2 and was obtained as a yellow oil in 57% yield; IR (NaCl film) ν_{max}: 3380.25 cm^{-1} (NH_2), 1747.97 cm^{-1} (β-lactam -C=O), 1676.35 cm^{-1} (-C=O); ^1H NMR (400 MHz, $CDCl_3$) δ 0.88 (d, 3H, J=7 Hz), 1.05 (d, 3H, J=7 Hz), 2.35 (m, 1H), 3.75-3.77 (m, 10H), 3.92 (s, 3H), 4.29-4.37 (m, 1H), 4.78-4.92 (m, 1H), 6.65 (s, 2H), 6.79 (s, 1H),

6.93 (m, 1H), 7.13 (m, 1H), 7.29-7.36 (m, 5H), 8.66 (d, 1H, J=13 Hz), 10.00 (d, 1H, J=13 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.2 (CH_3), 19.3 (CH_3), 30.4 (CH), 30.5 (CH), 55.1, 55.5 (OCH_3), 55.6 (OCH_3), 55.7 (OCH_3), 60.4 (OCH_3), 60.5 (OCH_3), 63.4 (C_3), 63.6 (C_3), 64.2 (C_4), 64.3 (C_4), 94.4 (CH, Ar), 94.5 (CH, Ar), 110.0 (CH, Ar), 110.2 (CH, Ar), 110.3 (CH, Ar), 111.0 (CH, Ar), 115.8 (CH, Ar), 117.5 (CH, Ar), 117.9 (CH, Ar), 120.0 (CH, Ar), 120.1 (CH, Ar), 126.9 (CH, Ar), 127.0 (CH, Ar), 127.3 (CH, Ar), 127.4 (CH, Ar), 127.5 (CH, Ar), 127.6 (CH, Ar), 128.3 (CH, Ar), 129.3 (CH, Ar), 129.4 (CH, Ar), 133.3 (C, Ar), 133.4 (C, Ar), 133.9 (C, Ar), 134.2 (C, Ar), 134.3 (C, Ar), 134.5 (C, Ar), 136.7 (C, Ar), 147.1 (C, Ar), 148.0 (C, Ar), 148.1 (C, Ar), 153.0 (C, Ar), 165.3 (C=O), 165.3 (C=O), 172.3 (C=O); HRMS: Calculated for $C_{30}H_{36}N_3O_6$: 534.2604; found 534.2584 $[M+H]^+$.

8.1.14. Synthesis of 7h, 10k-10m and 11p-11r

The carboxybenzyl or benzyl protected compound **7e**, **10e**, **10f**, **10j**, **11h**, **11n** or **11o** (2 mmol) was dissolved in ethanol: ethyl acetate (50 mL; 1:1 mixture) and hydrogenated over 1.2g of 10% palladium on carbon until complete on TLC, typically less than 2 hours. The catalyst was filtered, the solvent was evaporated under reduced pressure and the product was isolated by flash column chromatography (dichloromethane: methanol gradient, 100:0 to 90:10).

8.1.14.1. 2-Methoxy-5-(4-oxo-3-phenyl-1-(3,4,5-trimethoxyphenyl)azetidin-2-yl)phenyl 2-aminopropanoate (7h) was prepared from compound **7e** following the procedure above in 58% yield. IR (NaCl film) ν_{max}: 1748.1 (β-lactam -C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.54 - 1.56 (m, 3H, CH_3), 3.76-3.92 (m, 12H, 4 × OCH_3), 4.30-4.32

(m, 1H, H_3), 4.83-4.88 (m, 1H, H_4), 6.62 - 6.64 (m, 2H, ArH), 6.88 - 6.95 (m, 1H, ArH), 7.01-7.04 (m, 1H, ArH), 7.14-7.15 (m, 1H, ArH), 7.34-7.41 (m, 5H, ArH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.6 (CH_3), 56.0 (OCH_3), 56.1 (OCH_3), 60.9 (OCH_3), 63.5 (C_3), 63.8 (C_3), 64.9 (C_4), 65.0 (C_4), 94.8 (CH, Ar), 94.9 (CH, Ar), 111.1 (CH, Ar), 112.1 (CH, Ar), 113.0 (CH, Ar), 117.9 (CH, Ar), 120.6 (CH, Ar), 124.5 (CH, Ar), 127.4 (CH, Ar), 127.9 (CH, Ar), 128.0 (CH, Ar), 129.0 (CH, Ar), 129.1 (CH, Ar), 129.9 (CH, Ar), 130.5 (CH, Ar), 133.7 (C, Ar), 134.4 (C, Ar), 134.5 (C, Ar), 134.8 (C, Ar), 140.3 (C, Ar), 146.4 (C, Ar), 146.9 (C, Ar), 153.5 (C, Ar), 153.6 (C, Ar), 165.6 (C=O); HRMS: Calculated for $C_{28}H_{31}N_2O_7$: 507.2131; found 507.2152 $[M+H]^+$.

8.1.14.2. 2-Amino-N-(4-(2-(4-methoxyphenyl)-4-oxo-1-(3,4,5-trimethoxyphenyl)azetidin-3-yl)phenyl)-3-phenylpropanamide (10k) was prepared from **10e** following the procedure above as a yellow oil in 51% yield; IR (NaCl film) ν_{max}: 1742 cm^{-1} (β-lactam -C=O), 1684 cm^{-1} (-C=O); ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.82-2.88 (m, 1H, CH_2), 3.35-3.39 (m, 1H, CH_2), 3.79-3.85 (m, 13H, 4 × OCH_3, CH), 4.26 (s, 1H), 4.84 (d, 1H, J=2.5 Hz), 6.62 (s, 2H), 6.97 (d, 3H, J=8.5 Hz), 7.25-7.39 (m, 10H), 9.52 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 40.0 (CH_2), 54.4 (OCH_3), 55.6 (OCH_3), 56.2 (CH), 58.8 (CH), 60.5 (OCH_3), 63.5 (C_3), 64.2 (C_4), 94.4 (CH, Ar), 114.2 (CH, Ar), 119.7 (CH, Ar), 124.1 (CH, Ar), 126.6 (CH, Ar), 126.9 (CH, Ar), 127.6 (CH, Ar), 127.8 (CH, Ar), 128.1 (CH, Ar), 128.2 (CH, Ar), 128.3 (CH, Ar), 128.4 (CH, Ar), 128.7 (CH, Ar), 128.9 (CH, Ar), 129.3 (CH, Ar), 129.9 (CH, Ar), 133.2 (C, Ar), 133.9 (C, Ar), 136.2 (C, Ar), 136.9 (C, Ar), 153.0 (C, Ar), 159.4 (C, Ar), 159.5 (C, Ar), 164.7 (C=O), 165.3

(C=O), 171.7 (C=O), 174.0 (C=O); HRMS: Calculated for $C_{34}H_{36}N_3O_6$: 582.2604; found 582.2613 $[M+H]^+$.

8.2. Solubility measurement. Solubility testing was carried out on selected samples **7**, **7a**, **8**, **8a**, **9**, **9a**, **10**, **10g**, **10i**, **10k**, **10l**, **11**, **11j**, **11k**, **11l**, **11p** and **11q** as follows: An amount of compound approximately equal to 5 mg was accurately weighed and suspended in a corresponding amount of distilled water in a vial (Wheaton sample vials with rubber lined caps) to give a concentration of 1 mg/mL. This suspension was placed on a shaking plate (IKA® MTS 2/4 digital) and agitated at 300 shakes/minute for 24 hours. After 24 hours, the vials were removed from the shaking plate. The solution/suspensions were filtered and the filtrate was assayed by HPLC using the conditions described above for purity testing to determine the amount of compound in solution.

8.3. Biochemical Methods

8.3.1. Antiproliferative studies. All assays were performed in triplicate for the determination of mean values reported. Compounds were assayed as the free bases isolated from reaction. The human breast tumour cell line MCF-7 was cultured in Eagles minimum essential medium in a 95% O_2/5% CO_2 atmosphere with 10% fetal bovine serum, 2 mM L-glutamine and 100 µg/mL penicillin/streptomycin. The medium was supplemented with 1% non-essential amino acids. Cells were trypsinised and seeded at a density of 2.5×10^4 cells/mL in a 96-well plate and incubated at 37 ^oC, 95%O_2/5% CO_2 atmosphere for 24 h. After this time they were treated with 2 µL volumes of test

compound which had been pre-prepared as stock solutions in ethanol to furnish the concentration range of study, 1 nM–100 µM, and re-incubated for a further 72 h. Control wells contained the equivalent volume of the vehicle ethanol (1% v/v). The culture medium was then removed and the cells washed with 100 µL phosphate buffered saline (PBS) and 50 µL MTT (1 mg/mL solution in PBS) added. Cells were incubated for 2 h in darkness at 37 ^{o}C. At this point solubilization was begun through the addition of 200 µL DMSO and the cells maintained at room temperature in darkness for 20 min to ensure thorough colour diffusion before reading the absorbance. The absorbance value of control cells (no added compound) was set to 100% cell viability and from this graphs of absorbance versus cell density per well were prepared to assess cell viability and from these, graphs of percentage cell viability versus concentration of compound added were drawn.

8.3.2. Tubulin polymerisation assay. The effect of selected compounds on the polymerisation of purified bovine brain tubulin was determined spectrophotometrically by monitoring the change in turbidity. Lyophilised tubulin (Cytoskeleton, Denver, CO) was re-suspended in ice cold G-PEM buffer (80 mM PIPES pH 6.9, 0.5 mM $MgCl_2$, 1mM EGTA, 1 mM GTP, 10.2% (v/v) glycerol) and added to wells on a half volume 96 well plate containing the designated concentration of drug or vehicle. Samples were mixed well and tubulin assembly was monitored at $A_{340\ nm}$ at 30 sec intervals for 60 min at 37^{o}C in a Spectramax 340PC spectrophotometer (Molecular Devices). IC_{50} values were calculated at 30 mins using GraphPad Prism software (GraphPad Software, Inc.).

8.3.3. Immunofluorescence and confocal microscopy. For immunofluorescence, MCF-7 cells were seeded at 1×10^5 per well on BD falcon four well chamber glass slides (BD Biosciences, San Jose, USA. Cells were treated with vehicle [1% ethanol (v/v)]; **2**, **8a**, **9a**, **10k**, [100 nM] or **11l** [1 µM] for 16 h. Following treatment, cells were washed gently in PBS, fixed for 30 min in methanol at -20 ^{o}C. Following washes in PBST (PBS and 0.1% Triton-X-100), cells were blocked in 5% BSA diluted in PBST (blocking buffer). Cells were then incubated with mouse anti-tubulin (DM1A (Merck Chemicals Ltd); 1:20 for 1 h at room temperature. Following washes in PBST cells were incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse (Dakocytomation, UK); 1:100 for 1 h at room temperature. Following washes in PBST, the cells were mounted in Ultra Cruz Mounting Media (Santa Cruz Biotechnology, Santa Cruz, CA) containing 4,6-diamino-2-phenolindol dihydrochloride (DAPI). Confocal images were captured using the OLYMPUS 1X81 microscope coupled with OLYMPUS FLUOVIEW Ver 1.5 software. All images in each experiment were collected on the same day using identical parameters.

8.3.4. Determination of DNA content by flow cytometry. MCF-7 cells were seeded at 1×10^5 cells/cm^3. After 24 h, cells were treated with vehicle [1% ethanol (v/v)]; **2**, **7a**, **8a**, **9a**, **10k**, [100 nM] or **11l** [1 µM] for 48 h. Cells were harvested by centrifugation at 800 × g for 10 min. Cell pellets were re-suspended in PBS and fixed in 70% ethanol: PBS overnight at -20 ^{o}C. Following centrifugation cell pellets were re-suspended in PBS supplemented with 0.5 mg/ml RNase and 0.15 mg/ml propidium iodide (PI). Following a 30 min incubation at 37^{o}C in the dark the PI fluorescence was measured on a linear scale

using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The amount of PI fluorescence is directly proportional to the amount of DNA present in each cell. The relative content of DNA indicates the distribution of a population of cells throughout the cell cycle. For example, cells in G_0G_1 are diploid and have a DNA content of 2N. Cells with the G_2M phases have a DNA content of 4N, while cells in S-phase have a DNA content between 2N and 4N. Apoptotic cells are sub-diploid (<2N). Data collection was gated to exclude cell debris and cell aggregates. At least 10,000 cells were analysed per sample. All data were recorded and analysed using the CellQuest software (Becton Dickinson).

8.4. Computational Procedures. For ligand preparation, all compounds were built in Molecular Operating Environment 2011.10 (MOE 2011.10) [38] and energy minimised. Conformers of these compounds were generated in MOE 2011.10 using the conformation search option. The stochastic method was chosen with an iteration limit of 100. All other parameters were remained unchanged. For the receptor preparation, the PDB entry 1SA0 was downloaded from the Protein Data Bank (PDB) [33]. All waters were retained in both isoforms. Addition and optimisation of hydrogen positions for these waters was carried out using MOE 2011.10 ensuring all other atom positions remained fixed. Using the reported X-ray structure of tubulin co-crystallised with a colchicine derivative, DAMA-colchicine (PDB entry-1SA0), possible binding orientations of the β-lactam ligands were probed with the docking program MOE 2011.10. Docking was carried out using MOE 2011.10, using the DAMA-colchicine to identity the binding site. Each

conformation was subsequently docked using the Triangle Matcher placement methodology setting. All the default parameters remained unchanged.

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Supplementary information available: General methods for synthesis and experimental characterisation of compounds **8a**, **8b**, **9a**, **9b**, **10a-10d**, **10f-10j**, **10l**, **10m**, **11a-11c**, **11e-11k**, **11m-11r**. ^{1}H and ^{13}C spectra for representative β-lactams **7a** and **10g**. Molecular modelling: overlay of the docked structures of DAMA-colchicine and β-lactams **10**, **10k**, **11** and **11l** in the colchicine-binding site of tubulin.

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Table Captions

Table 1. Antiproliferative activities and physiochemical properties of β-lactam phosphate esters

[a]IC_{50} values are half maximal inhibitory concentrations required to inhibit the growth of MCF-7. Values represent the mean ± S.E.M for at least three independent experiments performed in triplicate. The IC_{50} value obtained for **2** in this assay was 5 nM for MCF-7 and is in good agreement with the reported values for **2** using the MTT assay on human MCF-7 breast cancer cell line [31, 39, 40].

[b]CLogP value calculated using ChemDraw Ultra and refers to $CLogP_{oct/water}$

[c]Solubility value taken as the maximum value from n=3, where each sample was assayed three times

[d]Solubility is below the level of detection of the assay

Table 2. Antiproliferative activity and solubility of β-lactam amino acid amides derived from **10**

[a]IC_{50} values are half maximal inhibitory concentrations required to inhibit the growth of MCF-7. Values represent the mean ± S.E.M for at least three independent experiments performed in triplicate

[b]CLogP value calculated using ChemDraw Ultra and refers to $CLogP_{oct/water}$

[c]Solubility value taken as the maximum value from n=3, where each sample was assayed three times

[d]Solubility is below the level of detection of the assay; nd=not determined

Table 3. Antiproliferative activity and solubility of β-lactam amino acid amides derived from **11**

[a]IC_{50} values are half maximal inhibitory concentrations required to inhibit the growth of MCF-7. Values represent the mean ± S.E.M for at least three independent experiments performed in triplicate.

[b]CLogP value calculated using ChemDraw Ultra and refers to $CLogP_{oct/water}$

[c]Solubility value taken as the maximum value from n=3, where each sample was assayed three times

[d]Solubility is below the level of detection of the assay; nd= not determined

[e]In murine 26 colon cells [21]

[f]Human plasma, pH 7.2-7.5 [21]

Figure captions

Figure 1. Phosphate prodrugs of tubulin-binding agents NAC, CA-4, CA-1 and phenstatin

Figure 2. Amino-acid prodrugs of amino analogues of CA-4 and CA-2

Figure 3. Antiproliferative β-lactams chosen for prodrug development. One enantiomer is illustrated.

Figure 4. Effects of compounds 7, 7a, 9 and 9a on the inhibition of tubulin polymerisation.

Effects of compounds **7, 7a, 9** and **9a** (10 μM) on *in vitro* tubulin polymerisation. Purified bovine tubulin and GTP were mixed in a 96-well plate. The reaction was started by warming the solution from 4 ºC to 37ºC. Ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340nm at 30 second intervals for 60 minutes at 37 ºC. The graph shows one representative experiment. Each experiment was performed in triplicate.

Figure 5. Effects of compound 11l on the inhibition of tubulin polymerisation.

Dose-response effect of compound **11l** on *in vitro* tubulin polymerisation. Purified bovine tubulin and GTP were mixed in a 96-well plate. The reaction was started by warming the solution from 4 ºC to 37ºC. Ethanol (1%v/v) was used as a vehicle control. The effect on tubulin assembly was monitored in a Spectramax 340PC spectrophotometer at 340nm at 30 second intervals for 60 minutes at 37 ºC. The graph shows one representative experiment. Each experiment was performed in triplicate.

Figure 6. Compound 2 and β-lactams 8a and 9a depolymerise the microtubule network of MCF-7 cells resulting in mitotic catastrophe. MCF-7 cells were treated with vehicle control (1% ethanol (v/v), A+E), or compound **2** (B+F), **8a** (C+G) or **9a** (D+H) (all 100 nM) for 16 h. Cells were fixed in methanol and stained with α-tubulin mAbs (green) and counterstained with DAPI (blue). Images were captured by confocal microscopy coupled with OLYMPUS FLUOVIEW software. Bar equal to 40 μm. Representative confocal micrographs of three separate experiments are shown.

Figure 7. Compound 2 and β-lactams 10k and 11l depolymerise the microtubule network of MCF-7 cells resulting in mitotic catastrophe. MCF-7 cells were treated with vehicle control (1% ethanol (v/v), A+D), compound **10k** (B+E; 100 nM) or **11l** (C,+F; 1 μM) for 16 h. Cells were fixed in methanol and stained with α-tubulin mAbs (green) and counterstained with DAPI (blue). Images were captured by confocal microscopy coupled with OLYMPUS FLUOVIEW software. Bar equal to 20 μm. Representative confocal micrographs of three separate experiments are shown.

Figure 8. Effects of 2 and β-lactams 7, 8, 9, 7a, 8a, 9a, 10k, 11l on the cell cycle. Percentages of cells in (A) sub-G1 and (B) G_2/M phase after treatment with β-lactams. MCF-7 cells were untreated (U), treated with vehicle control (V, 1% ethanol (v/v)), or compound **2**, **7**, **8**, **9**, **7a**, **8a**, **9a**, **10k** (100 nM) or **11l** (1 μM) for 48 h. Values represent the mean ± SEM for at least three independent experiments. Using one –way anova followed by Bonferroni's Multiple Comparison Test, there is no statistical difference between all β-lactam compounds for percentage of cells in sub-G1. G_2/M $p<0.001$ for all compounds compared to vehicle with no statistical difference between parent compounds and derivatives.

Figure 9. 2D representation of the binding interactions of (A) **10** and (B) **10k** with the colchicine binding site of tubulin.

Figure 10. 2D representation of the binding interactions of (A) **11** and (B) **11l** with the colchicine binding site of tubulin.

Scheme Captions

Scheme 1. Preparation of β-lactam phosphate esters 7a, 8a, 9a[a]

[a]Reagents and conditions: (a) Dibenzyl phosphite, dimethyl phosphite, or diethyl phosphite, DIPEA, DMAP, CCl_4, CH_3CN (b) Pd/C, H_2, EtOH:EtOAc (1:1) (c) (i) $BrSi(CH_3)_3$, CH_2Cl_2 (ii) $Na_2S_2O_3$. (Bn = $-CH_2C_6H_5$)

*Only one enantiomer is illustrated

Scheme 2. Synthesis of β-lactams 7e-7l[a]

[a]Reagents and conditions: (a) DCC, DMAP (b) H_2, Pd, EtOh, EtOAc (c) Et_3N, $C_6H_5CH_2COCl$, CH_2Cl_2 (d) $BrCH_2CH_2Br$, $(nBu)_4NHSO_4$, NaOH; (e) Et_3N, $C_6H_5CH_2COCl$, CH_2Cl_2; (f) $MeNH_2$

Scheme 3. Preparation of β-lactams 10a-10j and 11a-11o[a]

[a]Reagents and conditions: (a) *N*-protected L-amino acid, anhydrous DMF, DCC (polymer supported), $HOBt.H_2O$, stirring, rt; (b) TBAF, 1-octanethiol, CH_2Cl_2, rt

Scheme 4. Preparation of β-lactams 10k-10m and 11p-11r[a]

[a]Reagents and conditions: (a) H_2, Pd/C, EtOAc: EtOH 1:1, rt

Table 1.

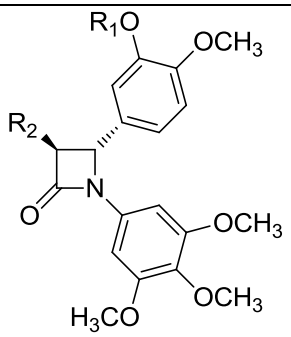
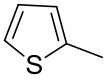
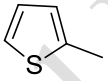
			**IC_{50} MCF-7 (nM)[a]**	**CLogP[b]**	**Solubility (mg/mL)[c]**
	R_1=	**R_2=**			
7	H	C_6H_5	9.6 ± 2.6[4]	3.14	0[d]
7a	$P(O)(OH)_2$	C_6H_5	71 ± 35	2.09	0.30
8	H		7.0 ± 2.0[5]	2.79	0[d]
8a	$P(O)(OH)_2$		20 ± 8	1.74	0.45
9	H	*p*-C_6H_4OH	0.8 ± 0.4[4]	2.47	0[d]
9a	$P(O)(OH)_2$	*p*-C_6H_4OH	22 ± 11	1.43	0.14

Table 2.

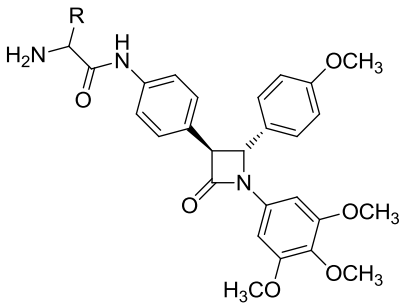
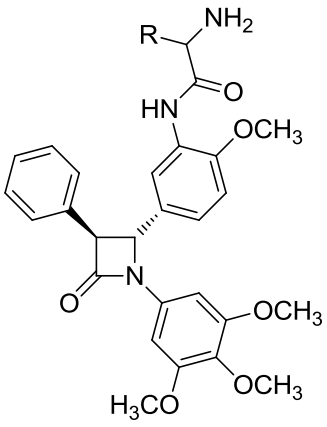
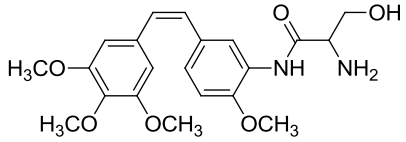
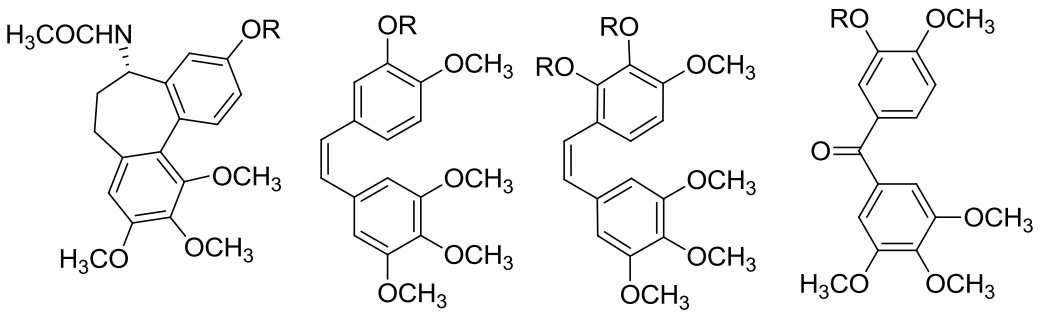
	Structure	**Amino acid**	**IC_{50} (nM)**[a]	**CLogP**[b]	**Solubility (mg/mL)**[c]
					
	R=				
10g	H	Gly	91 ± 10	1.99	0.06
10h	CH_3	Ala	35 ± 20	2.30	nd
10i	$(CH_2)_4NH_2$	Lys	690 ± 700	2.00	0[d]
10k	$CH_2C_6H_5$	Phe	27 ± 0.3	3.71	0.14
10l	$CH(CH_3)_2$	Val	50 ± 20	3.22	0.004
10m	CH_2OH	Ser	860 ± 300	1.13	nd
10	Parent compound		50 ± 20 [4]	2.65	0

Table 3.

	Structure	Amino acid	IC_{50} MCF-7 (nM)[a]	CLogP[b]	Solubility (mg/mL)[c]
					
	R=				
11i	H	Gly	1100 ± 900	1.55	nd
11j	CH_3	Ala	760 ± 230	1.86	0.37
11k	$CH_2C_6H_5$	Phe	1130 ± 500	3.28	0[d]
11l	$CH(CH_3)_2$	Val	460 ± 200	2.79	0.11
11m	$(CH_2)_4NH_2$	Lys	9800 ± 2000	1.56	nd
11p	$CH(CH_3)C_2H_5$	Ile	1100 ± 900	3.32	0.11
11q	CH_2OH	Ser	780 ± 200	0.70	0.20
11r	$CH_2C_6H_4OH$	Tyr	740 ± 60	2.61	nd
11	Parent compound (Figure 3)		650 ± 10 [4]	3.00	0[d]
5a		Ser	37[e]	0.87	0.11[f]



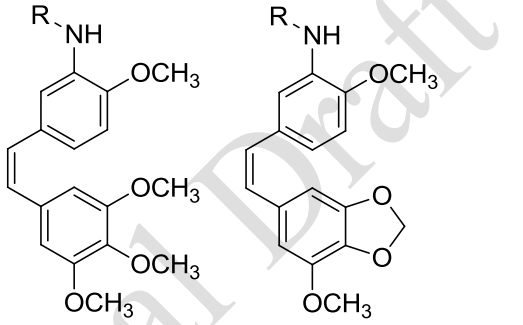
1 (NAC): R=H
1a: R=P(O)OH_2

2 (CA-4): R=H
2a: R=P(O)$(ONa)_2$

3 (CA-1): R=H
3a: R=P(O)$(ONa)_2$

4: R=H
4a: R=P(O)ONaOH

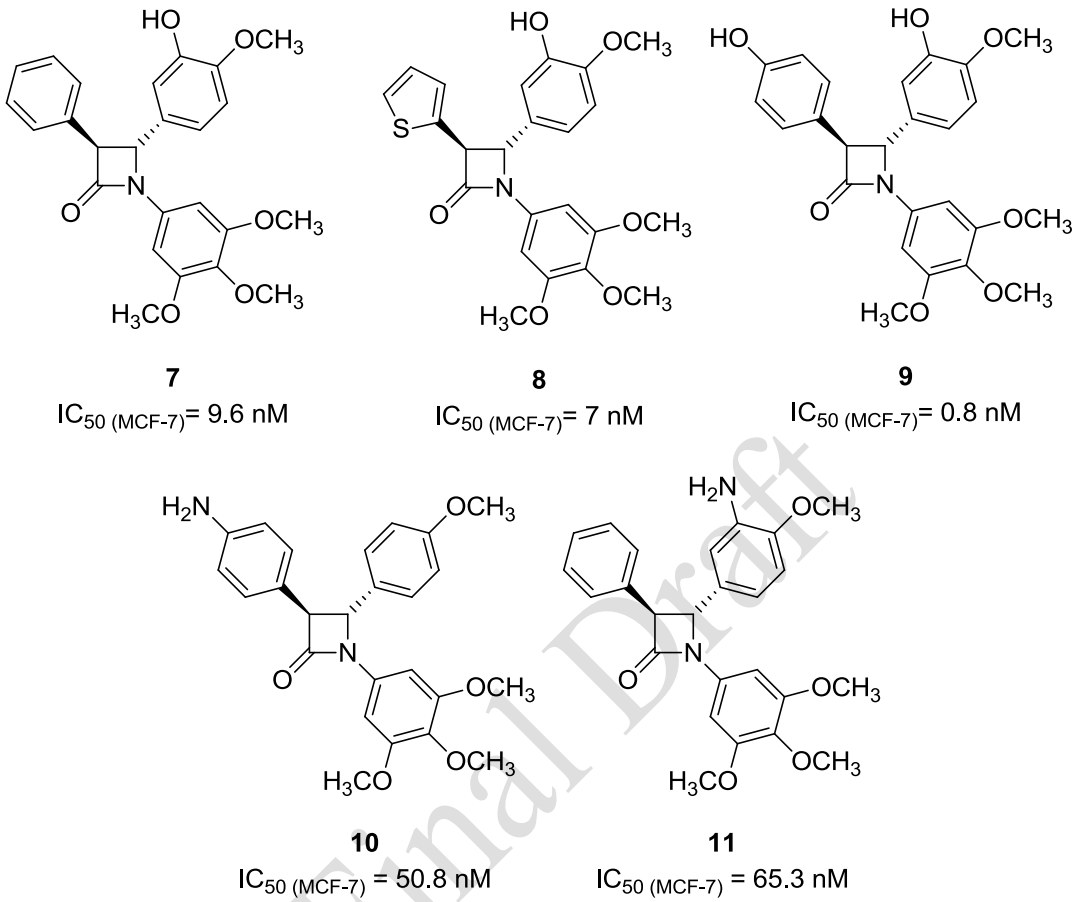
Figure 1.



5: R=H
5a: R=C(O)$CH(CH_2OH)(NH_2)$

6: R=H
6a: R=C(O)CH_2NH_2

Figure 2.



7
$IC_{50\ (MCF\text{-}7)}$= 9.6 nM

8
$IC_{50\ (MCF\text{-}7)}$= 7 nM

9
$IC_{50\ (MCF\text{-}7)}$= 0.8 nM

10
$IC_{50\ (MCF\text{-}7)}$ = 50.8 nM

11
$IC_{50\ (MCF\text{-}7)}$ = 65.3 nM

Figure 3.

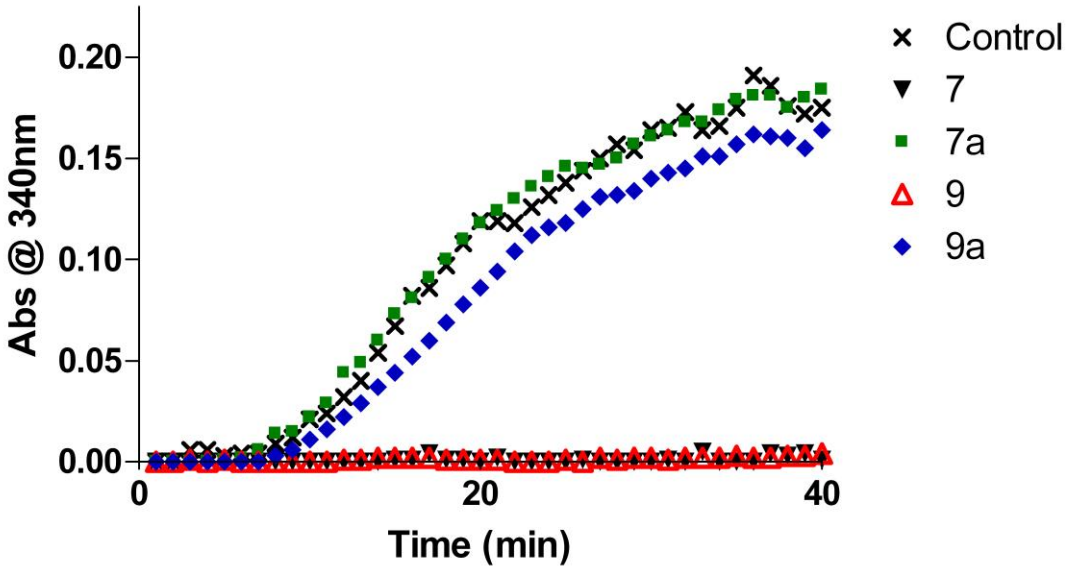


Figure 4.

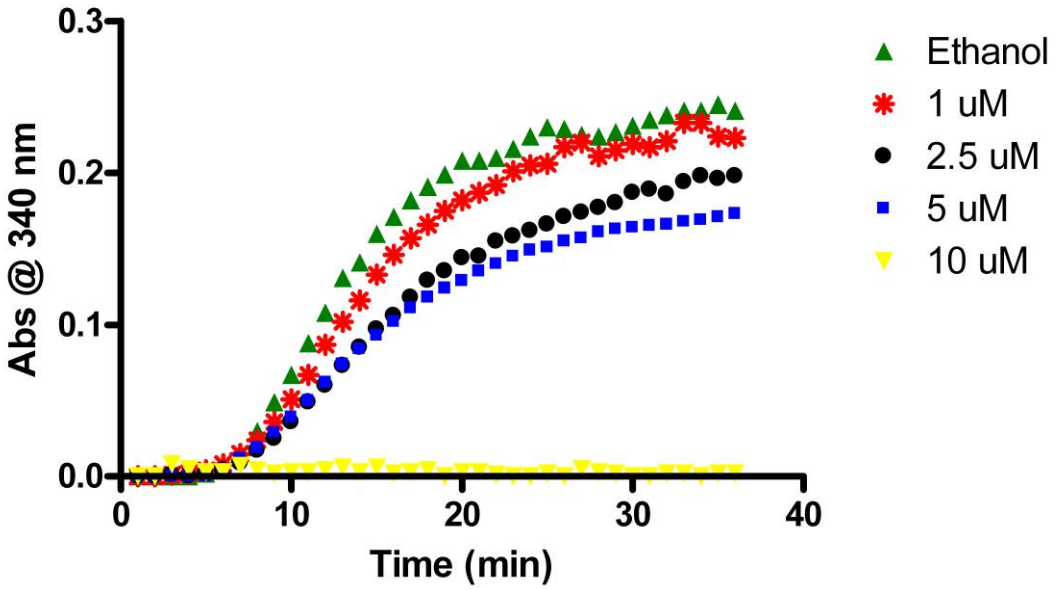


Figure 5.

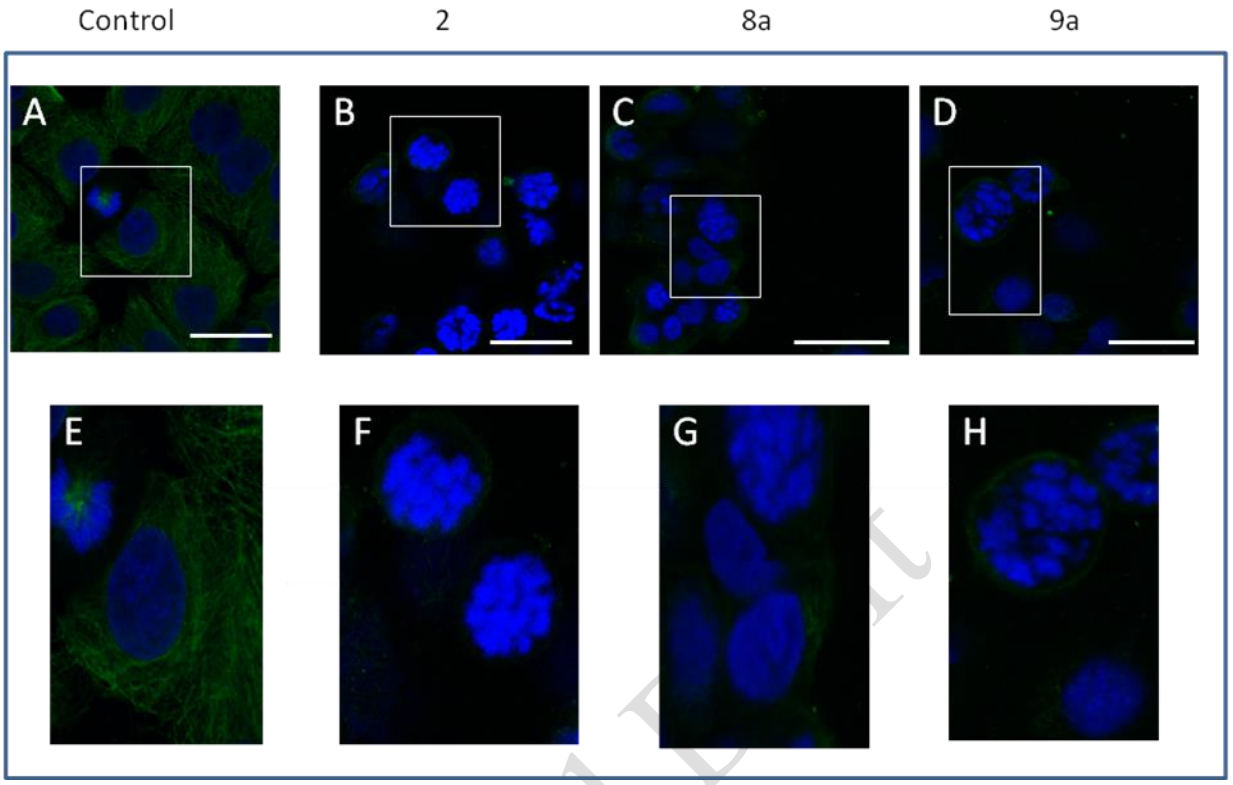


Figure 6.

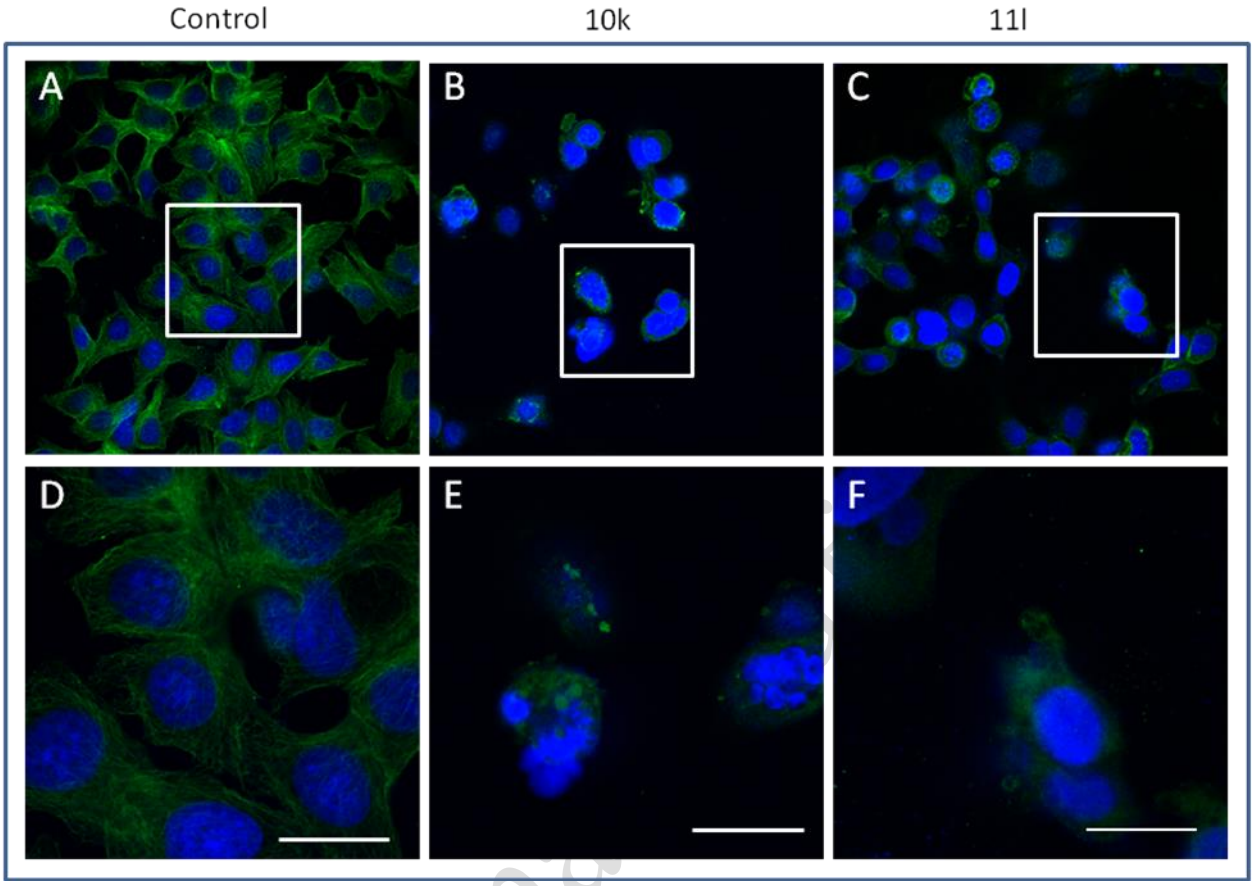


Figure 7.

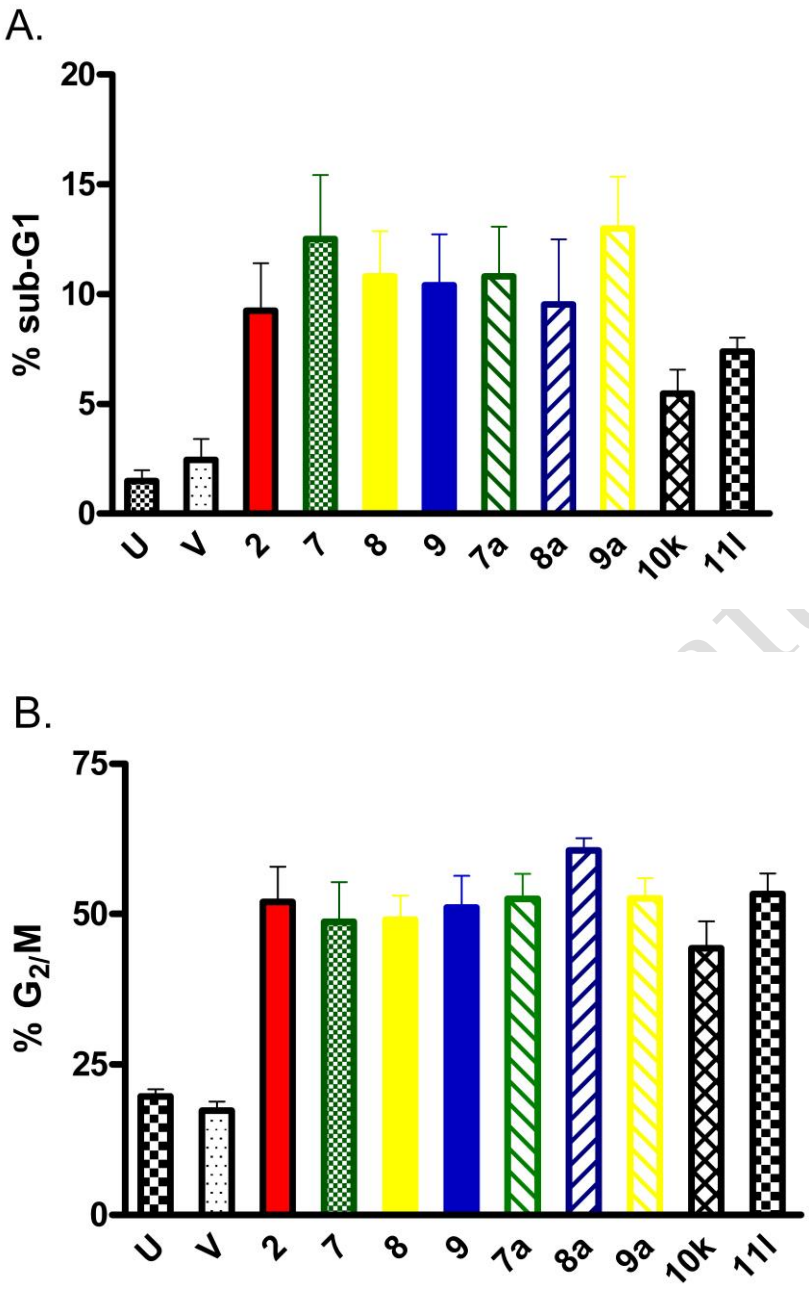
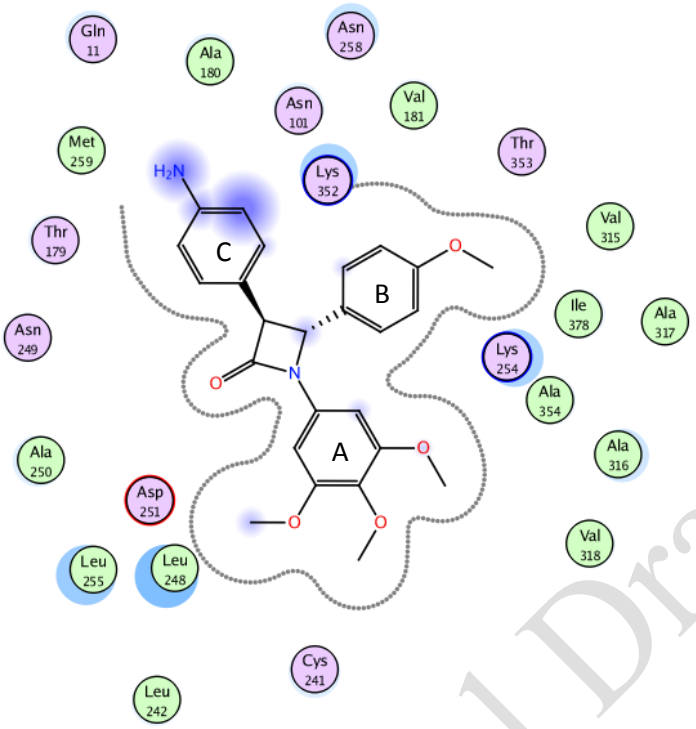

A.
% sub-G1
20
15
10
5
0
U
V
2
7
8
9
7a
8a
9a
10k
11l
B.
% G2/M
75
50
25
0
U
V
2
7
8
9
7a
8a
9a
10k
11l

Figure 8.

A.



B.

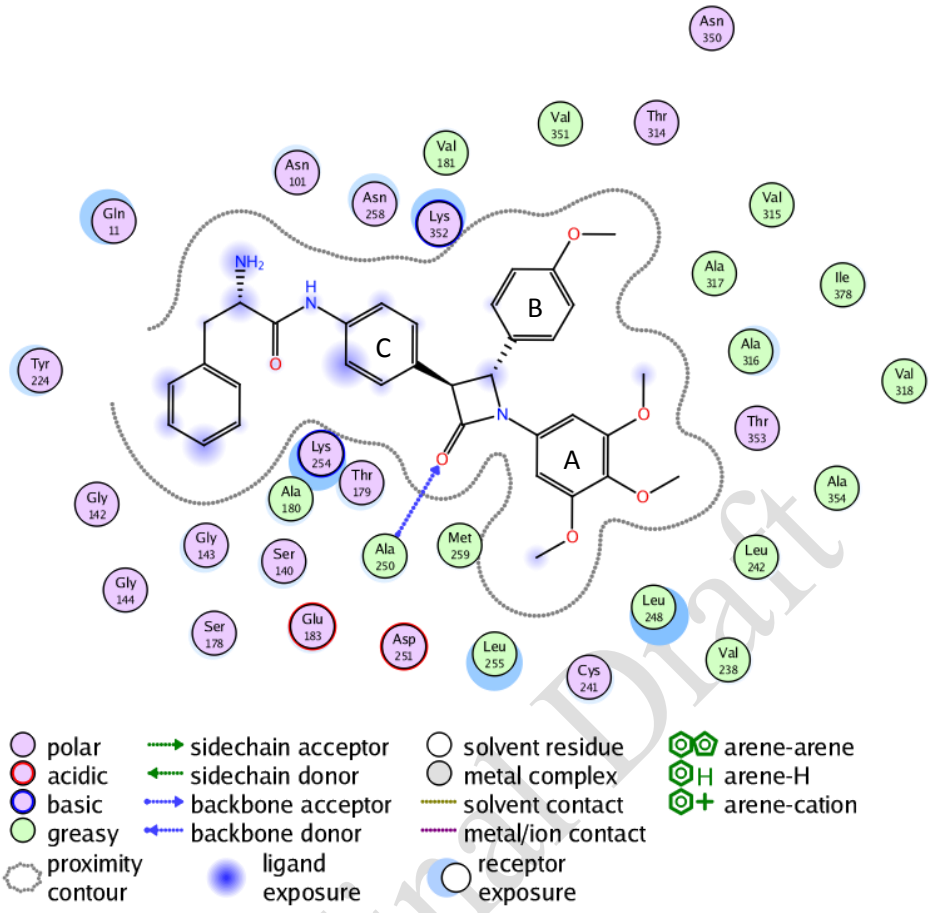
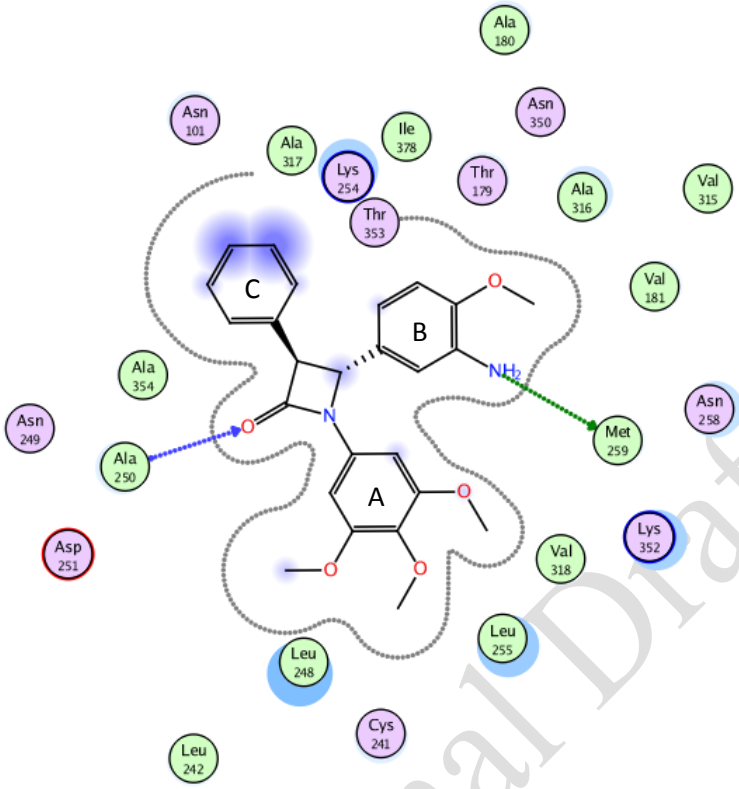


Figure 9.

A.



B.

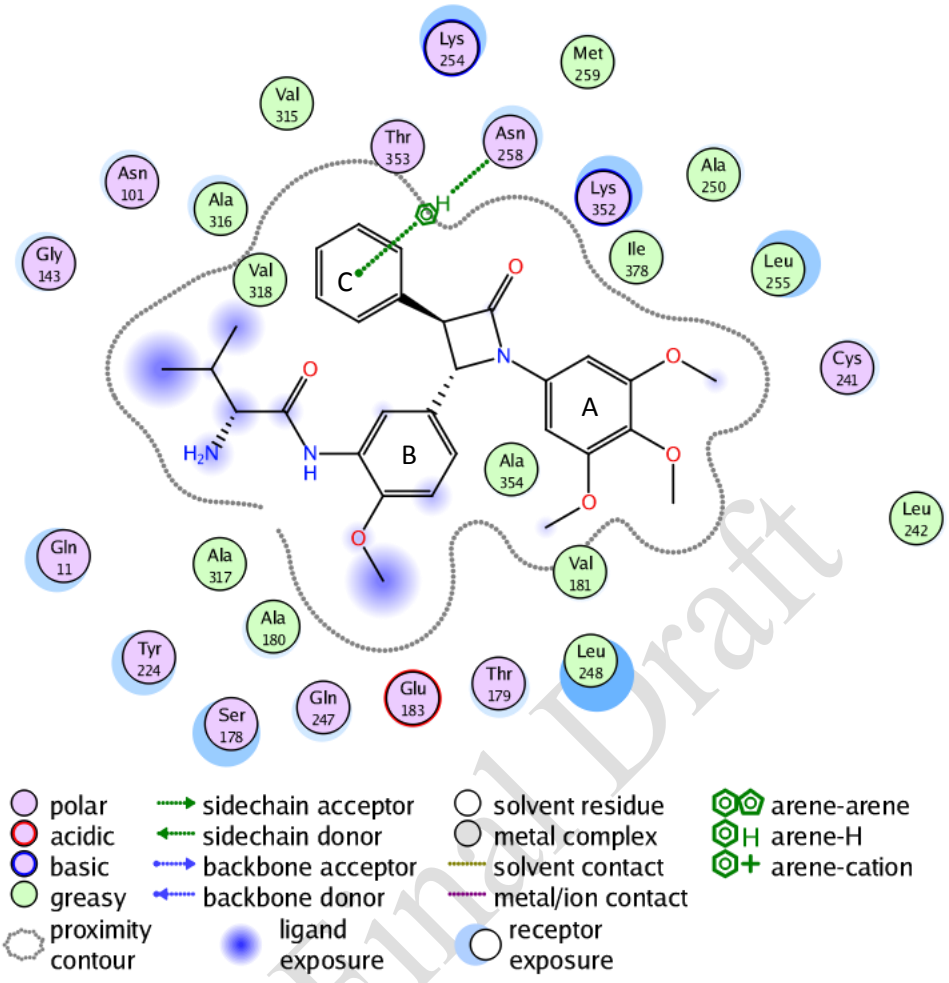
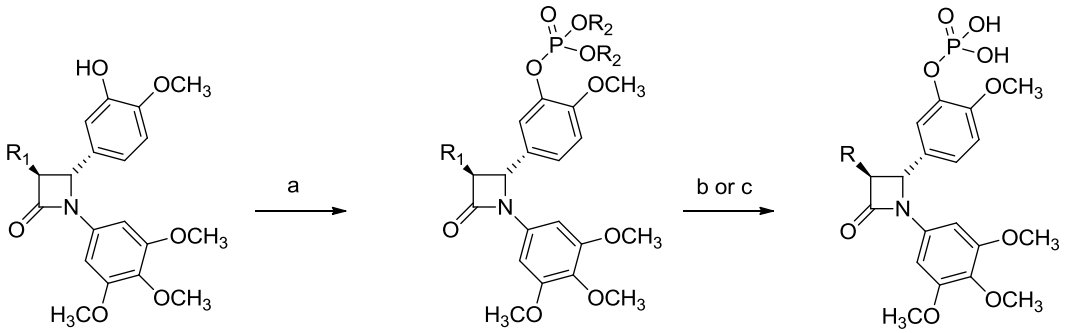


Figure 10.

Schemes

Scheme 1.

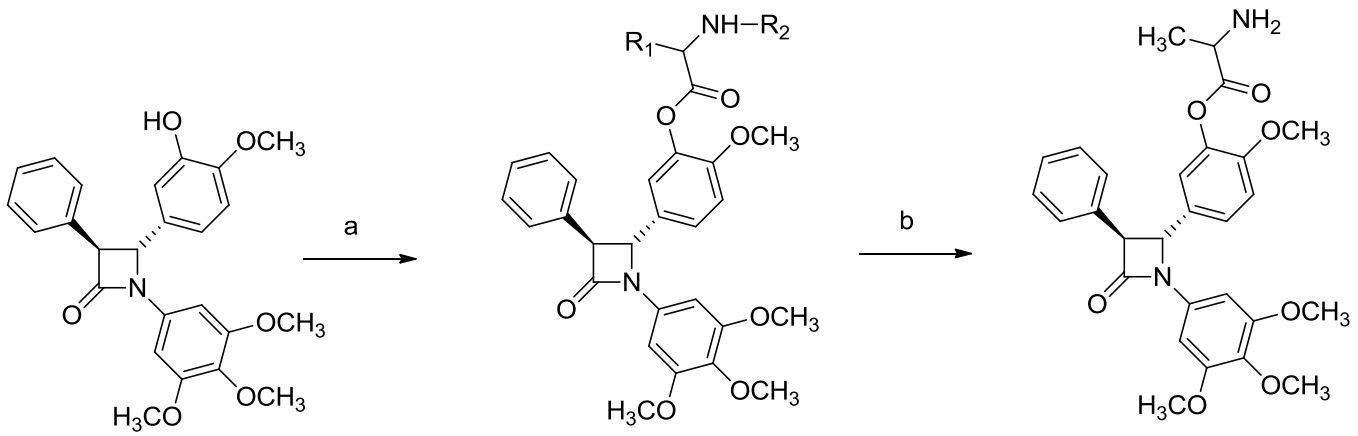


7: R=C_6H_5
8: R=C_4H_3S
9c: R=4-$BnOC_6H_4$

7b: R_1=C_6H_5, R_2=Bn
8b: R_1=$C_6H_5C_4H_3S$, R_2=Bn
9b: R_1= 4-$BnOC_6H_4$, R_2=Bn

7a: R=C_6H_5
8a: R=C_4H_3S
9a: R=4-C_6H_4OH

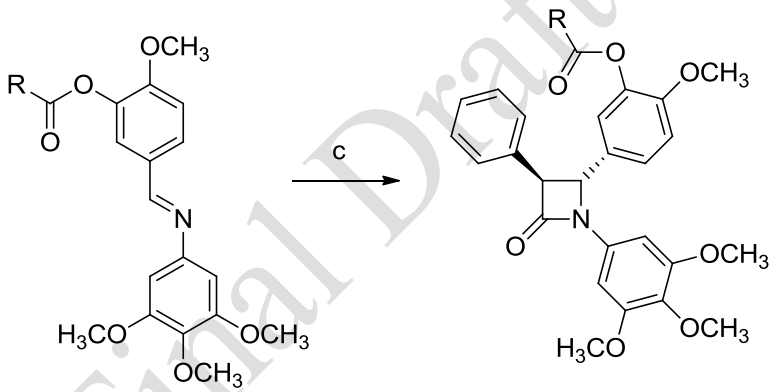
Scheme 2.



7

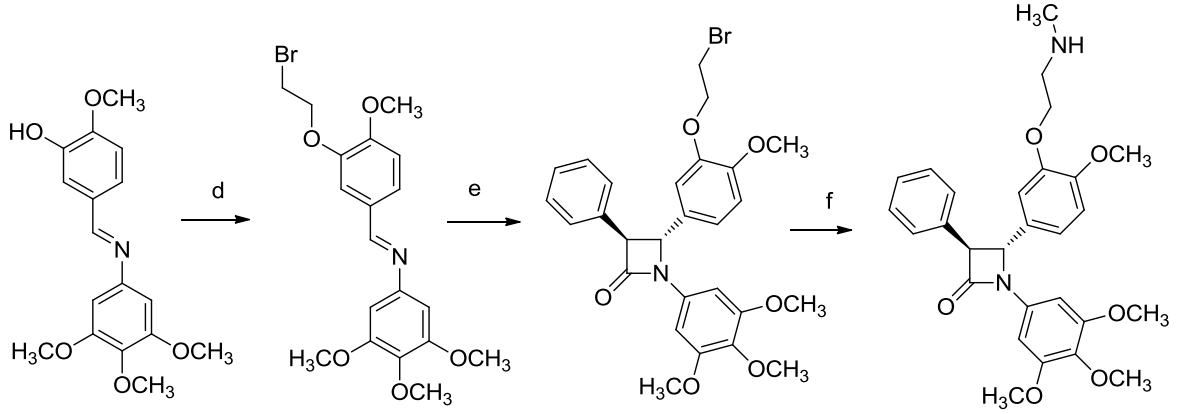
7e: R_1= CH_3, R_2 = Cbz
7f: R_1= $CH_2C_6H_5$, R_2 = Cbz
7g: R_1= H, R_2 = $COCH_3$

7h



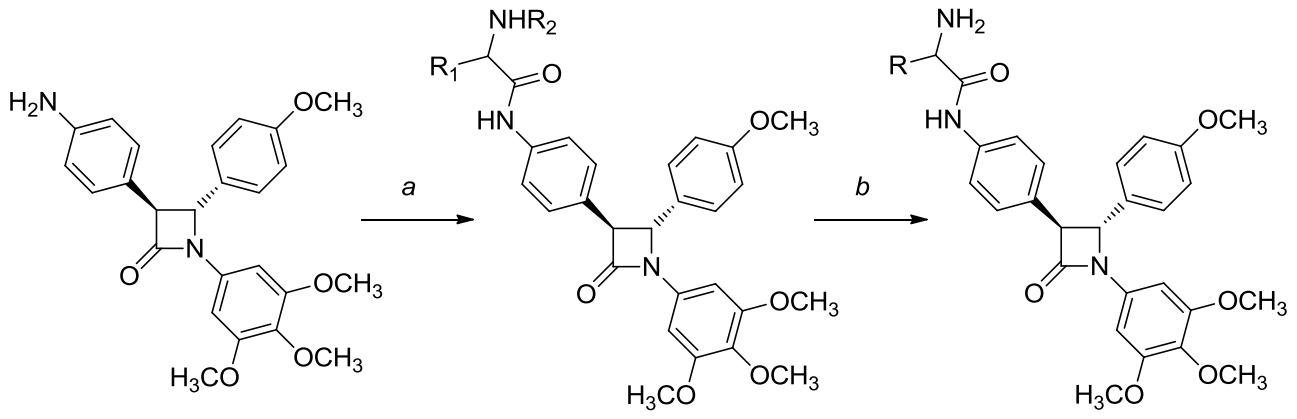
12a: R = CH_3
12b: R = C_6H_5

7i: R = CH_3
7j: R = C_6H_5



12c **12d** **7k** **7l**

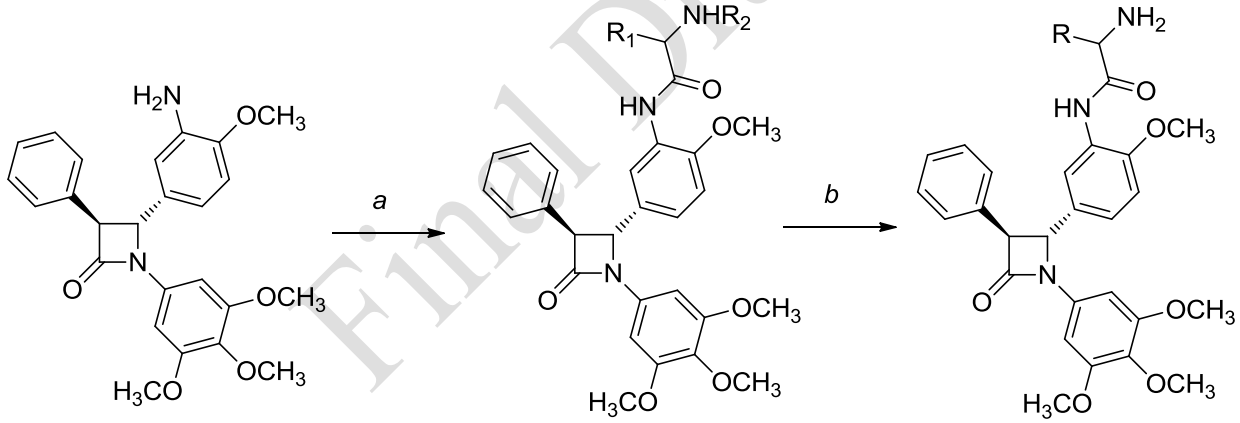
Scheme 3.



10

10a: R_1=H; R_2=Fmoc
10b: R_1=CH_3; R_2=Fmoc
10c: R_1=$(CH_2)_4NHFmoc$; R_2=Fmoc
10d: R_1=$CH_2OCH_2C_6H_5$; R_2=Fmoc
10e: R_1=$CH_2C_6H_5$; R_2=Cbz
10f: R_1=$CH(CH_3)_2$; R_2=Cbz

10g: R_1=H
10h: R_1=CH_3
10i: R_1=$(CH_2)_4NH_2$
10j: R_1=$CH_2OCH_2C_6H_5$

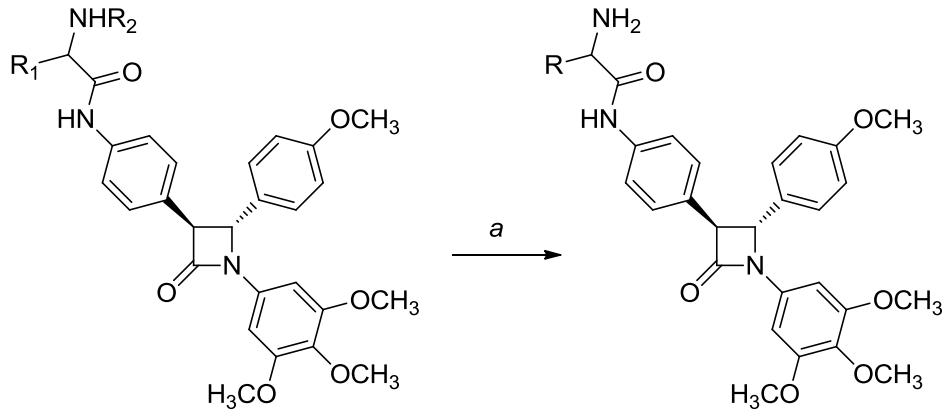


11

11a: R_1=H; R_2=Fmoc
11b: R_1=CH_3; R_2=Fmoc
11c: R_1=$CH_2C_6H_5$; R_2=Fmoc
11d: R_1=$CH(CH_3)_2$; R_2=Fmoc
11e: R_1=$(CH_2)_4NHFmoc$; R_2=Fmoc
11f: R_1= $CH_2OCH_2C_6H_5$; R_2=Fmoc
11g: R_1=$CH_2C_6H_4OCH_2C_6H_5$; R_2=Fmoc
11h: R_1=$CH(CH_3)C_2H_5$; R_2=Cbz

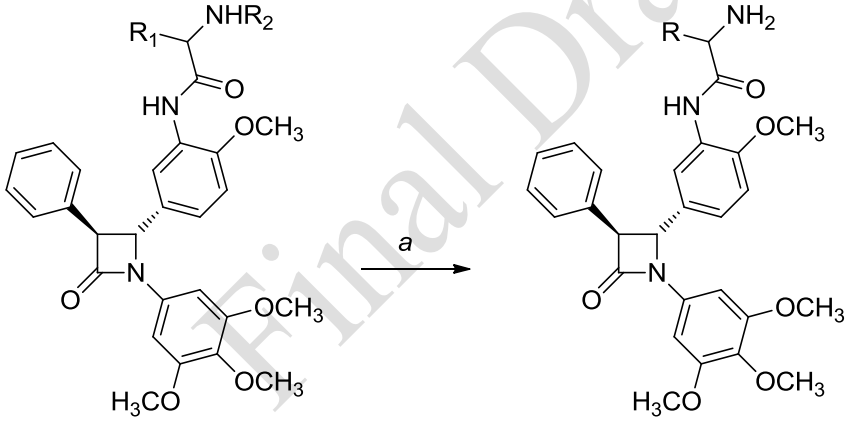
11i: R_1=H
11j: R_1=CH_3
11k: R_1=$CH_2C_6H_5$
11l: R_1=$CH(CH_3)_2$
11m: R_1=$(CH_2)_4NH_2$
11n: R_1=$CH_2OCH_2C_6H_5$
11o: R_1=$CH_2C_6H_4OCH_2C_6H_5$

Scheme 4.



10e: $R_1=CH_2C_6H_5$; R_2=Cbz
10f: $R_1=CH(CH_3)_2$; R_2=Cbz
10j: $R_1=CH_2OCH_2C_6H_5$; R_2=H

10k: $R=CH_2C_6H_5$
10l: $R=CH(CH_3)_2$
10m: $R=CH_2OH$



11h: $R_1=CH(CH_3)C_2H_5$; R_2=Cbz
11n: $R_1=CH_2OCH_2C_6H_5$
11o: $R_1=CH_2C_6H_4OCH_2C_6H_5$

11p: $R=CH(CH_3)C_2H_5$
11q: $R=CH_2OH$
11r: $R=CH_2C_6H_4OH$